Master of Science

Anti-melanogenic effect of Bay 11-7082 on post-inflammatory hyperpigmentation (PIH)

Post-inflammatory hyperpigmentation (PIH)에 대한

Bay 11-7082 의 멜라닌생성 저해 효과

The Graduate School

of the University of Ulsan

Department of Medical Science

Juwon Moon

Anti-melanogenic effect of Bay 11-7082 on post-inflammatory hyperpigmentation (PIH)

Supervisor : Sung Eun Chang

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Juwon Moon

Department of Medical Science

University of Ulsan, Korea

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This certifies that the master's thesis of Juwon Moon is approved.

Committee Chair Dr. Youngsup Song

Committee Member Dr. Sung Eun Chang

Committee Member Dr. Ik Jun Moon

Department of Medical Science

University of Ulsan, Korea

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Abstract

Post-inflammatory hyperpigmentation (PIH) refers to an acquired pigmentation disorder that causes pigmentation due to inflammation of the skin due to external causes such as injury or skin disease. It is a serious problem that affects skin health and appearance. Various treatments have been developed to deal with this problem, but there is still no complete cure. Additionally, there is a lack of understanding of the occurrence mechanism and underlying physiological causal relationship of PIH. The molecular understanding of how inflammation affects melanogenesis and how melanin production changes after inflammation is still unknown territory. Therefore, by analyzing the mechanism of PIH, we aim to understand the interaction between inflammation and melanin production and develop an effective treatment that simultaneously controls inflammation and melanin production.

UVB is one of the ultraviolet rays emitted from sunlight and activates the MAPK pathway, promoting MITF and its subordinate genes Tyrosinase, Trp1, and DCT, which are involved in melanin production. This study confirmed that Bay 11-7082 inhibits melanin increased by Forskolin (FSK) or UVB in melanocytes, mouse skin, and ex vivo human skin. Bay 11-7082 inhibits cellular tyrosinase activity increased by FSK and MAPK, it was confirmed that the levels of MITF, Tyrosinase, DCT, and Trp1 were reduced. In addition, it suppressed the levels of cytokines IL-1a, IL-1b, IL-6, TNF-a, COX-2, and MCP-1, and induced inflammation in mice with DNFB, causing pigmentation and inflammation and inflammation by Bay 11-7082. It was confirmed that melanin was reduced.

As a result, Bay 11-7082 is an NF-κB inhibitor that reduces inflammation by suppressing cytokines and suppresses melanin through the MAPK/MITF pathway, so it can simultaneously suppress inflammation and melanin in the development of a treatment for PIH. It is judged to have great potential as a functional raw material.

(Keywords) Post-inflammatory hyperpigmentation (PIH), Bay 11-7082, MAPK/MITF pathway, melanogenesis.

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Introduction

Melanogenesis

Human skin color is determined by complex genetic factors and environmental influences [1]. Melanin is a factor that determines the color of our skin, eyes, and hair, and the diversity of pigmentation appears due to changes in the number and type of melanocytes, the size and number of melanosomes, and differences in transmission and distribution between keratinocytes and melanocytes. Additionally, melanin protects the skin from ultraviolet rays by absorbing ultraviolet rays.

Melanin is produced in melanosomes, which are organelles of melanocytes, and melanosomes move into the dendrite of melanocytes. Melanosomes that move to dendrites move from the basal layer to the stratum corneum. Melanosomes are absorbed into keratinocytes and move to the periphery of the nucleus, forming a melanin cap that protects DNA from ultraviolet rays [2]. This determines the color of the skin. Keratinocytes break down after a certain period and are replaced by new cells. Through this process, pigmentation does not occur, and the individual's original skin color returns.

Cells surrounding melanocytes, such as keratinocytes, fibroblasts, and immune cells, affect melanin production by secreting paracrine factors in response to ultraviolet irradiation or inflammatory factors. Additionally, several studies have shown that the extracellular matrix (ECM), which is involved in signaling pathways in skin cells, can affect melanin production [3].

The main cause of melanin production is UV. When affected by UV, melanin gathers in the nucleus of keratinocytes to protect the skin from UV damage and protects DNA damage. However, when UV is excessively irradiated, the transcription factor p53 is activated due to DNA damage in keratinocytes, and p53 binds to the promoter of the pro-opiomelanocortin (POMC) gene, thereby inducing the expression of POMC [4]. Alpha-melanocyte stimulating hormone (α-MSH) is secreted by the expressed POMC, and α-MSH binds to the melanocortin 1 receptor (MC1R) of melanocytes in a paracrine manner. Activated MC1R dissociates subunit α of G protein, activating adenylyl cyclase (AC) [5], which converts ATP (Adenosine triphosphate) into cyclic adenosine monophosphate (cAMP), increasing the concentration of cAMP within the cell. An increase in cAMP activates PKA (protein kinase A). Activated PKA enters the nucleus and is phosphorylated at serine 133 of cAMP response element protein (CREB), a transcription factor for MITF. Phosphorylated CREB increases the transcriptional activity of CREB target genes such as MITF. After the generated MITF is phosphorylated and dimerized with p300,

it synthesizes Tyrosinase, Tyrp-1, and DCT, which are called melanin-related enzymes, as a transcription factor complex, increasing melanogenesis and causing pigmentation [6].

Melanin synthesis

Melanin is divided into eumelanin, a yellowish-orange type of pigment, and pheomelanin, a brownish-blackish pigment, and is synthesized in melanosomes present within melanocytes. Tyrosinase is involved in melanin synthesis by producing L-3-(3,4-dihydroxyphenyl)-alanine (L-DOPA) from the amino acid L-tyrosine. The produced L-DOPA is oxidized to L-DOPA quinone by tyrosinase. This L-DOPA quinone is synthesized into eumelanin by proteins called tyrosinase-related protein 1 (Tyrp-1) and dopachrome tautomerase (DCT) or converted to pheomelanin in the presence of cysteine [7]. Additionally, the pH of melanosomes also affects the determination of eumelanin and pheomelanin [8].

Melanosomes that produce melanin move from the cell membrane of the dendrite tip of melanocytes to surrounding keratinocytes [9] through exocytosis, cytophagocytosis, and plasma membrane fusion [10]. In the migrated melanosome, melanin is located around the nucleus to the DNA of keratinocytes caused by UV [11]. The expression of Tyrosinase, Tyrp-1, and DCT enzymes involved in melanin synthesis is regulated on the mRNA of microphthalmia-associated transcription factor (MITF) [12].

Post-inflammatory hyperpigmentation (PIH)

Post-inflammatory hyperpigmentation (PIH) is an acquired pigmentation disorder that causes pigmentation due to skin inflammation or external causes such as injury or skin disease. It is a serious problem that affects skin health and appearance [13].

PIH pathogenesis usually occurs through interaction between keratinocytes and melanocytes. When exposed to external injury or ultraviolet rays, keratinocytes release inflammatory cytokines such as IL-1, IL-6, IL-10, and TNF-α, and these cytokines increase Tyrp-1 and DCT, which are related to melanogenesis [14].

Additionally, dermatitis occurs when inflammatory cells move to skin tissue and secrete substances such as inflammatory cytokines. As a result, the affected melanocytes move from the epidermis to the dermis, causing pigmentation through melanin synthesis in the dermis [15]. Additionally, melanin is

absorbed into cells through the phagocytosis of inflammatory cells in the dermis, which is called melanophage. These macrophages deposit in the dermis, causing pigmentation [16].

However, although various treatments for PIH have been developed, there is still no complete cure. Additionally, there is a lack of understanding of the occurrence mechanism and underlying physiological causal relationship of PIH. The molecular understanding of how inflammation affects melanin synthesis and how melanogenesis changes after inflammation is still unknown.

MAPK/MITF pathway

Mitogen-activated protein kinase (MAPK) includes Extracellular signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38 MAPK. These MAPKs respond within cells to external stimuli and play an important role in extracellular signaling such as proliferation, apoptosis, differentiation, stress response, and development [17]. Phosphorylation of ERK, JINK, and p38 MAPK leads to MITF ubiquitination and degradation and inhibits melanin production [18]. Therefore, we show that MAPK is involved in the regulation of MITF expression and activation and is one of the key regulators of melanin biosynthesis. Inhibition of ERK leads to an increase in melanin production, while activation of p-ERK phosphorylates MITF at Ser 73, leading to ubiquitination and proteasome-mediated degradation of MITF, which is associated with the downregulation of melanogenesis [19]. JNK is only involved in the activity of CRTC, not CREB activity, and inhibition of JNK causes dephosphorylation and nuclear translocation of CRTC, which leads to the expression of MITF and increases melanin [20]. p38 phosphorylation inhibits tyrosinase melanin production through protein degradation and is involved in stress-induced melanin production [21].

Bay 11-7082

Bay 11-7082 is known to be an anti-inflammatory compound that inhibits the activation of NF-κB by inhibiting the phosphorylation of IκB-α [22].

NF-κB is an important transcription factor widely involved in the regulation of apoptosis, inflammation, immunity, and cell proliferation [23]. It encodes proteins involved in immune regulation but also regulates the transcription of genes important for cell survival, differentiation, and proliferation of non-immune cells. Therefore, abnormal activity of NF-κB plays an important role in many diseases of the immune system [24]. NF-κB subunits of p65, p50, c-Rel, Rel B, and p100/52 exist as homodimers or

heterodimers depending on the cell. In macrophages such as Raw 264.7, p65 and p50 exist as heterodimers. When lipopolysaccharide (LPS) binds as a ligand for Toll-like receptor 4 (TLR4), reactive oxygen species (ROS) are generated, and these ROS activate the IκB kinase (IKK) complex. The activated IKK complex phosphorylates IκB bound to NF-κB, causing ubiquitination. This occurs when the E3 ligase enzyme binds ubiquitin to the target protein. The bound protein moves to a cellular organelle called the proteasome, where the target protein is broken down into amino acids. NF-κB, separated from IκB, moves to the nucleus, and acts as a transcription factor, increasing the expression of inflammatory cytokines such as COX-2, MCP-1, TNF-α, IL-1, and IL-6. In this pathway, Bay 11-7082 inhibits IκB phosphorylation and inhibits NF-κB from acting as a transcription factor.

According to a recent study, Bay 11-7082 is known to block the production of NO, PGE2, and TNF-α in LPS-treated Raw 264.7 cells [25] and to suppress substances related to inflammatory responses such as iNOS and COX-2 [26]. Additionally, it inhibited the proliferation and growth of uveal melanoma cells by inducing apoptosis without affecting the cell cycle [27]. Therefore, in addition to these research results, Bay 11-7082 is known for many research results related to inflammation inhibition, and it is expected to affect melanin reduction by strongly suppressing inflammation-related substances. If melanin decreases simultaneously with inflammation, it can be used as an agent for inflammatory skin diseases such as pigmentation and acne caused by UV rays. However, Bay 11-7082 has not been studied on melanin synthesis.

Therefore, we used the LOPAC library (Sigma-Aldrich; St. Louis, MO) to find drugs that simultaneously reduce inflammation and melanin for the treatment of PIH. First, screening was conducted to select drugs that reduce melanin through melanin content assay in B16F10 cells. As a result, about 10 drugs were selected. At the same time, to confirm the inhibition of the activity of NF-κB, which plays an important role in controlling inflammation, a luciferase assay of NF-κB-Luciferase Reporter (Luc) - Jurkat cells was performed. Among the 10 drugs, the results showed that Bay 11-7082 reduced NF-κB activity the most. It was confirmed that the inhibition of melanin synthesis was due to the activation of ERK and JNK which resulted in a decrease in melanin due to a decrease in the expression of MITF and Tyrosinase. In addition, by inducing inflammation in K14-SCF mice with DNFB and treating them with Bay 11-7082, simultaneous reduction of inflammation and melanin was confirmed.

Materials and Methods

1. Compounds

Bay 11-7082 ((E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile, Sigma-Aldrich, St. Louis, MO, USA)) and **forskolin** (FSK, MedChemExpress, NJ, US) are used by dissolving in DMSO (Dimethyl sulfoxide). **Lipopolysaccharides** from Escherichia coli (LPS, Sigma-Aldrich, St. Louis, MO, USA) were used by dissolving in DW (Distilled water).

2. Cell line and cell culture

Mouse Melanocyte (Mel-ab) cells (KCLB, Seoul, South Korea) from a mouse-derived spontaneously immortalized melanocyte cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM, WelGENE, Daegu, South Korea) supplemented with 10% Fetal bovine serum (FBS, WelGENE), 1% Antibiotic-Antimycotic (AA, Grand Island, NY, USA), 100 nM 12-o-tetradecanolyphorbol-13-acetate (TPA, Sigma-Aldrich, St. Louis, MO, USA) and 1 nM Cholera toxin (Sigma-Aldrich, St. Louis, MO, USA). **Normal human melanocytes (NHM)** (Invitrogen, Carlsbad, CA, USA) cells were cultured in Medium 254 (Gibco, Cascade Biologics, Portland, OR, USA) supplemented with 1% AA and Human Melanocyte Growth Supplement (HMGS, Invitrogen, Carlsbad, CA, USA) and **Normal human keratinocytes (NHK)** (KCLB, Seoul, South Korea) cells were cultured in EpiLife™ (Gibco, Cascade Biologics, Portland, OR, USA) with 1% AA and Human Keratinocyte Growth Supplement (HKGS, Invitrogen, Carlsbad, CA, USA). **Raw 264.7 (**KCLB, Seoul, South Korea) and **Hacat** (ATCC, Manassas, VA, USA) **cells** were cultured in an incubator at 37°C and 5% CO2 using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% AA. **NF-kB Luciferase Reporter Jurkat Stable cells** were cultured in an incubator at 37°C and 5% CO2 using RPMI 1640 (WelGENE, Daegu, South Korea) supplemented with 10% FBS and 1% AA**.**

3. Luciferase assay

NF-kB Luciferase reporter Jurkat stable cells were placed in a 96-well plate at 1 x 10^5 cells per well. The drug to be screened (1 μM) and PMA (Sigma-Aldrich, St. Louis, MO, USA) (10nM) was dispensed

and incubated for 4 h in an incubator at 37°C and 5% CO2 conditions. Then, it was centrifuged at 1200 rpm for 1 min and the supernatant was removed. 100 μl of Luciferase lysis buffer (1 X gly-gly, 1% TritonX-100, 1 mM DTT) was added to the pellet and stored overnight in a deep freezer. Then, it was melted on ice and centrifuged at 1200 rpm for 1 min. 25 μl of cell lysate and 25 μl of Assay mixture (1 X gly-gly, 1 M Pota-phosphate pH 7.8, 100 mM ATP, 1 M DTT) were added to a white 96-well plate, and 50 μl of Luciferin was dispensed. Then, luminescence was measured using a Multi-Mode Microplate reader (BioTek, Winooski, Vermont, USA).

4. Cell viability assay

To confirm cell viability, it was evaluated using MTT reagent (2.5mg/ml) (Sigma-Aldrich, Germany). Mel-ab cells were seeded at 3×10^5 and NHM at 6×10^5 in each well of a 6-well plate and cultured in an incubator at 37°C and 5% CO2 for 24 h. After 24 h, the medium was changed 1 h before drug treatment, and Bay 11-7082 was treated at different concentrations. After 72 h, before harvesting, the MTT reagent was treated and incubated in an incubator at 37°C and 5% CO2 for 1 h. The medium was suctioned, 1 ml of DMSO per well was added, and the formazan crystals were dissolved in a shaker at room temperature for 10 minutes. Afterward, Cell viability was measured at an absorbance of 565 nm with the microplate reader (BioTek, Winooski, Vermont, USA).

5. Melanin content and tyrosinase activity assay

To confirm the change in melanin content according to drug concentration, Mel-ab cells were seeded in the number of 3×10^5 and NHM was seeded in the number of 6×10^5 in each well of a 6-well plate. Cells were cultured for 24 h in an incubator at 37°C and 5% CO2 conditions. After 24 h, the medium was changed 1 h before drug treatment, Bay 11-7082 was treated at each concentration, and Forskolin (10 μM) was pre-treated for 1 hour. After 72 hours of drug treatment, the cells were washed twice with cold 1X PBS (Phosphate-buffered saline), and then 550 μl of 1N NaOH was added to lyse the cells. It was boiled at 100°C for 30 minutes and vortexed twice in between. After 30 min, centrifugation was performed at 13,000 rpm for 5 min, and measured at an absorbance of 405 nm with the microplate reader. Bradford assay was performed to use the same protein, and this value was used to calculate the melanin content with the same amount of protein.

To confirm the change in Tyrosinase activity according to the treatment of Bay 11-7082, Mel-ab cells were seeded in the number of 3×10^5 and NHM were seeded in the number of 6×10^5 in each well of a 6-well plate. Cells were cultured for 24 h in an incubator at 37°C and 5% CO2 conditions. After 24 h, the medium was changed 1 hour before drug treatment, Bay 11-7082 was treated at 5 μM, and Forskolin (10 μM) was pre-treated for 1 h. After 72 h of drug treatment, the cells were washed in ice-cold 1X PBS and lysed in 300 μl of Tyrosinase lysis buffer (0.1M sodium phosphate pH6.8, containing 1% Triton X-100) with freeze/thaw cycles. The lysates were centrifuged at 15,000 rpm at 4°C for 10 min. To quantify the protein levels of the lysate, the Bradford assay was performed using the supernatant. Using this value, each sample was adjusted to the same amount of protein, and then 90 μl was dispensed into a 96-well plate, and 10 μl of L-DOPA (10 nM) (L-3,4-dihydroxyphenylalanine) was added to each well. Incubation was conducted at 37°C and 5% CO2, and tyrosinase activity was measured by reading the absorbance at 475 nm every 10 min for 1 h using a microplate reader.

6. Western blot

After washing with cold 1X PBS, cells were harvested by dispensing a protein lysis buffer (1% SDS in 10 mM Tris-HCI pH8.0) into each well. 5X SDS-PAGE loading buffer was dispensed and incubated at 98°C for 10 min. it was stored at -20°C.

Extracted protein samples were separated by 8% SDS-polyacrylamide gel electrophoresis, and it performed at 65 V for 20 min and 90 V for 85 min. Afterward, transfer was performed at 0.2 A for 90 minutes using nitrocellulose membranes (NC membrane, 0.45 μM, Amersham, Marlborough, MA, USA). After washing with 1X TBST (containing 0.1% Tween 20), three times for 10 minutes each, blocking was performed with 5% Bovine Serum Albumin (BSA) for 1 h. Afterward, the primary antibody was diluted in 5% BSA, added to the membrane, and reacted overnight on a shaker at 4°C.

MITF, Tyrosinase, Tyrp1, and DCT used as primary antibodies were purchased from abcam (Cambridge, UK). The antibodies against the total and phosphor form of ERK, p38 MAPK, and SAPK/JNK were purchased from Cell Signaling Technology (Danvers, MA, USA). HSP90α/β was purchased from Santa Cruz (Dallas, TX, USA) and used as an internal loading control. Each primary antibody was diluted 1:1000. After washing with 1X TBST, the secondary antibody was reacted with 5% skimmilk at 1:4000 on a shaker for 1 h. Next, the secondary antibody was removed after washing with 1X TBST. The membrane was reacted using SuperSignal™ West Pico PLUS Chemiluminescent Substrate and developed on X-ray film (AGFA, CP-BU new) in a dark room.

7. Quantitative Real-Time PCR

The total cellular RNA was extracted from Mel-ab cells using the FavorPrep™ Total RNA Purification Mini Kit (FAVORGEN Biotech, Taiwan). The concentration of extracted RNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA was diluted to 2ug/ml using Nuclease Free water. After diluted RNA was incubated at 65°C for 5 minutes, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL). 8 µl of Fast SYBRTM Green Master Mix, 3 µl of DW, 2 ug/ml cDNA, and 10 pmole/µl of forward and reverse primer were dispensed into each well of LightCycler® 480II Multiwell Plate 96, white (Roche, Basel, Switzerland). A quantitative real-time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) using a LightCycler® 480II machine. The cycle used at this time was pre-incubation at 95°C for 5 minutes, Ramp Rate at 4.4°C/s, Amplification at 95°C for 10 seconds, Ramp Rate at 4.4°C/s at 60°C for 10 seconds, Ramp Rate at 2.2°C/s, 72°C single acquisition mode for 15 seconds, Ramp Rate was set to 4.4°C/s and 40 cycles were performed. The melting curve was set to 95°C for 5 seconds, Ramp Rate 4.4°C/s, 65°C for 1 minute, Ramp Rate 2.2°C/s, 97°C Continuous, Acquisitions 5 per °C. Cooling was set to 40°C for 30 seconds and 2.2°C/s Ramp Rate.

Primer	Sequence
mGAPDH-F	5’-CAT CAC TGC CAC CCA GAA GAC TG-3’
mGAPDH-R	5’-ATG CCA GTG AGC TTC CCG TTC AG-3’
mMITF-F	5’-GGG ATG CCT TGT TTA TGG TG-3’
mMITF-R	5’-CAC CGC AGA CCA CTT AGT CC-3’
mTyrosinase-F	5’-TTA TGC GAT GGA ACA CCT GA-3’
mTyrosinase-R	5’-GAG CGG TAT GAA AGG AAC CA-3’
mTyrp1-F	5’-CCC CTA GCC TAT ATC TCC CT-3
mTyrp1-R	5’-TAC CAT CGT GGG GAT AAT GG-3’
mDCT-F	5’-CTT TGC AAC CGG GAA GAA CG-3’
mDCT-R	5’-CCG ACT AAT CAG CGT TGG GT-3’
IL-1α-F	5’-ACG GCT GAG TTT CAG TGA GAC C-3’
IL-1α-R	5’-CAC TCT GGT AGG TGT AAG GTG C-3’
IL-1β-F	5’-TGG ACC TTC CAG GAT GAG GAC A-3’
IL-1β-R	5’-GTT CAT CTC GGA GCC TGT AGT G-3’

IL-4-F	5’-ATC ATC GGC ATT TTG AAC GAG GTC-3’
IL-4-R	5’-ACC TTG GAA GCC CTA CAG ACG A-3’
IL-6-F	5’-TAC CAC TTC ACA AGT CGG AGG C-3’
IL-6-R	5’-CTG CAA GTG CAT CAT CGT TGT TC-3’
IL-8-F	5’-GGT GAT ATT CGA GAC CAT TTA CTG-3’
IL-8-R	5’-GCC AAC AGT AGC CTT CAC CCA T-3’
IL-10-F	5’-CGG GAA GAC AAT AAC TGC ACC C-3’
IL-10-R	5’-CGG TTA GCA GTA TGT TGT CCA GC-3’
TNF-α-F	5’-AGT TCT ATG GCC CAG ACC CT-3’
TNF-α-R	5’-GTC TTT GAG ATC CAT GCC GT-3’
COX-2-F	5’-GCG ACA TAC TCA AGC AGG AGC A-3’
COX-2-R	5’-AGT GGT AAC CGC TCA GGT GTT G-3’
MCP-1-F	5’-GCT ACA AGA GGA TCA CCA GCA G-3’
MCP-1-R	5’-GTC TGG ACC CAT TCC TTC TTG G-3’

Table 1. List of primers used for quantitative real-time PCR.

8. *Ex vivo* skin organ culture

Human skin tissues were obtained from the Department of Plastic Surgery, Asan Medical Center, Seoul (Institutional Review Board (2020-0091)). The fat was removed, washed with 70% EtOH, 100% EtOH, and PBS, and then cut into 2 cm^2 pieces. Afterward, DMEM containing 10% AA and 10% FBS was dispensed into a 6-well plate, a sterilized wire mesh was placed, and the tissue was placed on it. Bay 11-7082 (50 μM) diluted in 30% propylene glycol and 70% EtOH was applied to the tissue, the medium was treated with Bay 11-7082 (25 μM) for 96 h, and the epidermis of the tissue was treated with UVB 220 mJ/ cm^2. The medium was changed every 24 hours and cultured in an incubator at 37°C and 5% CO2 for 96 h.

9. Fontana-Masson staining

Skin tissue was fixed in 10% formalin. Fixed human skin tissues were made into paraffin blocks and slides were made into 6 μM-thick sections. To remove paraffin, it was placed in a dry oven at 60°C for

1 h. After that, it was dipped twice in 100% xylene for 10 min each, then twice in 100% EtOH for 5 minutes each, then dipped in 95% EtOH, 80% EtOH, and 70% EtOH for 5 minutes each, and placed in water for washing. The ammoniacal silver solution was dropped on the tissue and placed in a 60°C dry oven until the tissue turned brown. Afterward, staining was performed using the Fontana-Masson kit (ID labs, London, ON, Canada) according to the instructions. To Confirm the staining condition through a microscope and dehydrate the tissue, it was dipped in 70% EtOH, 80% EtOH, and 95% EtOH for 3 seconds each, and then placed in 100% Xylene for 1 min. After covering it with a cover slide, melanin was observed through a microscope. The melanin index was measured as the percentage of stained area relative to the total tissue area using ImageJ software (version 1.53K, National Institutes of Health).

10. Animals

C57BL6/J (000664) and K14-SCF transgenic mice (009687) were purchased from Jackson Laboratories (USA). These two types of mice were crossed and the K14-SCF mice were used. To induce inflammation, the hair was removed, and the abdomen was sensitized with 0.5% DNFB. Two days later, the back was pretreated with 100 μl of Bay 11-7082 (30 μM) for 1 h. Treatment was performed four times every other day in the same manner. Afterward, 100 μl of 0.1% DNFB was treated. Then, 100 μl of Bay 11-7082 (30 μM) was treated every other day for 26 days. Ears and tails were treated with 100 μl each of Bay 11-7082 (30 μM) for 35 days.

11. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM), and statistical analysis was performed using the GraphPad Prism (version 8, San Diego, CA USA) program. The graph was averaged using SEM. p values <0.05, <0.01 and <0.001 were considered statistically significant and marked with *, ** and ***, respectively.

Result

Inhibitory effect of Bay 11-7082 on inflammatory cytokines.

To find a drug that can reduce melanin synthesis and inflammation at the same time, the LOPAC library was used to select drugs that showed a melanin-reducing effect in B16F10. Then, NF-κB-Luciferase Reporter (Luc) - Jurkat Cell was used to find drugs that regulate the NF-κB signaling pathway, an important transcription factor widely involved in the regulation of apoptosis, inflammation, immunity, and cell proliferation.

Screening was performed by treating these cells with PMA to increase NF-κB- luciferase (NF-κB-Luc) reporter genes and by treating them with drugs that have a melanin reduction effect. As a result, it was confirmed that Bay 11-7082 reduced NF-κB-luciferase (NF-κB-Luc) reporter genes (Figure 1).

When IκB is phosphorylated in the cytoplasm of Raw 264.7 cells stimulated with LPS, p65 bound to IκB is phosphorylated and moves into the nucleus to act as a transcription factor. Since p65 phosphorylation increases substances such as inflammatory cytokines and is involved in their movement from the cytoplasm to the nucleus [28]. We confirmed whether Bay 11-7082 inhibits p65 phosphorylation as an NF-κB inhibitor. Raw 264.7 cells were treated with Bay 11-7082 at 0.1 – 10 μM to confirm cytotoxicity, and there was no cytotoxicity up to 10 μM. (Figure 2). It was confirmed that p65 phosphorylation was inhibited by Bay 11-7082 (Figure 3). NF-κB increases the expression of inflammatory cytokines such as COX-2, MCP-1, TNF-α, IL-1, and IL-6. In this pathway, Bay 11-7082 inhibits the phosphorylation of IκB, thereby inhibiting both moving translocation to the nucleus of NF-κB and acting as a transcription factor [29]. Therefore, we confirmed whether Bay 11-7082, an NF-κB inhibitor, reduces inflammation by suppressing the expression of inflammatory cytokines. To confirm the mRNA expression of inflammatory cytokines such as IL-6, IL-1, TNF-α, MCP-1, and COX-2, which are direct transcriptional target genes of NF-κB, 5 μM of Bay 11-7082 was added to Raw 264.7 cells. After treatment for 1 hour, LPS 100ng/ml was treated for 2 h. As a result, COX-2, MCP-1, TNF-α, IL-1, and IL-6 were inhibited by Bay 11-7082 (Figure 4). Additionally, Hacat cells were used to confirm the inhibition of skin inflammation. Cytotoxicity was confirmed by treating Hacat cells with Bay 11-7082 at 0.1 – 10 μM, and there was no cytotoxicity up to 10 μM (Figure 5). Hacat cells were pretreated with Bay 11-7082 (5uM) for 1 h and then treated with UVB 100 mJ/ cm^2. After 2 h, it was confirmed that the expression of inflammatory cytokine mRNA such as COX-2, TNF-α, IL-1, and IL-6 was suppressed by Bay 11-7082 (Figure 6).

Inhibitory effect of Bay 11-7082 on melanin synthesis.

Mel-ab cells were used to confirm the inhibitory effect of Bay 11-7082 on melanin synthesis. To evaluate cell viability, Mel-ab cells were treated with 0.1 - 10 μM at each concentration for 72 h. Cell viability was measured at an absorbance of 565 nm with the microplate reader. As a result, it was confirmed that there was no cytotoxicity up to 5 μM (Figure 7).

It was confirmed whether Bay 11-7082 inhibits melanin synthesis. When Mel-ab cells were treated alone with Bay 11-7082 at a non-toxic concentration of 0.1 μM to 5 μM for 72 h, it decreased by more than 20% in a concentration-dependent manner starting from 0.1 μM. When exposed to UVR, melanin synthesis is induced by the cAMP-PKA-CREB signaling system, so FSK, which acts as a cAMP agonist, was treated at 10 μM, and Bay 11-7082 was pre-treated at each concentration for 1 h. After 72h, Melanin was measured. As a result, when treated with 10 μM of FSK, it decreased in a concentration-dependent manner, and at 5 μM, the effect of suppressing melanin synthesis by more than 40% was shown (Figure 8). It is confirmed under the microscope (Figure 9, 10).

Since Bay 11-7082 reduced melanin synthesis, it was expected to reduce the activity of tyrosinase, which directly affects melanin synthesis, so Mel-ab cells were pre-treated with 5 μM of Bay 11-7082 for 1 hour before treatment with 10 μM of FSK. Tyrosinase activity was confirmed after 72 h. As expected, a decrease in tyrosinase activity was observed when treated with Bay 11-7082 (Figure 11).

Reduction of melanin synthesis through inhibition of melanogenesis-related protein expression in Bay 11-7082.

To confirm that the inhibition of melanin synthesis by Bay 11-7082 was due to changes in the expression of melanin synthesis-related proteins, Mel-ab cells were treated with 5 μM of Bay 11-7082 alone or pre-treated with 5 μM of Bay 11-7028 for 1 hour before treatment with 10 μM of FSK. After treatment, the cells were harvested at 48 and 72 hours to observe the protein expression levels of MITF, Tyrosinase, Tyrp-1, and DCT. As a result, it was confirmed that the expression of melanin synthesis-related proteins was reduced by Bay 11-7082 when treated with Bay 11-7082 alone and when treated with FSK (Figure 12). To confirm changes in the expression of genes related to melanin synthesis, when treated with Bay 11-7082 alone and FSK at 24 and 48, mRNA levels were not significantly reduced compared to protein levels (Figure 13).

We confirmed whether the decrease in melanin synthesis-related proteins was due to proteasome-mediated protein degradation. Mel-ab cells were treated with MG132 (150 nM), a proteasome inhibitor, for 2 h before treatment with Bay 11-7082 (5 μM). FSK treatment was pre-treated for 12 h. After 72h, it was shown that Bay 11-7082 inhibits melanin synthesis through the degradation of melanin synthesis-related proteins (Figure 14), and it was confirmed that melanin is regulated by proteasome-mediated protein degradation.

Reduction of melanin through increased phosphorylation of ERK, JNK, and p38 by Bay 11-7082 treatment.

Mitogen-activated protein kinase (MAPK) includes Extracellular signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38 MAPK. These MAPKs respond within cells to external stimuli and play an important role in extracellular signaling such as proliferation, apoptosis, differentiation, stress response, and development. Phosphorylation of ERK, JINK, and p38 MAPK leads to MITF ubiquitination and degradation and inhibits melanin production. Therefore, we show that MAPK is involved in the regulation of MITF expression and activation and is one of the key regulators of melanin biosynthesis. Therefore, to confirm that the inhibition of melanin synthesis by Bay 11-7082 was due to the activation of ERK, JNK, and P38, we treated Mel-ab cells with 5 μM of Bay 11-7082 for 30 – 120 min. As a result, it was observed that Bay 11-7082 activated ERK, JNK, and P38 (Figure 15). Since activation of ERK, JNK, and P38 seemed to reduce melanin synthesis, PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (P38 inhibitor) were used to determine whether the ERK, JNK, and P38 signaling pathway is involved in melanin production. After pre-treatment for 1 hour, Bay 11-7082 and FSK were treated simultaneously. As a result, it was confirmed that the melanin content reduced by Bay 11-7082 was restored by ERK, JNK, and P38 inhibitors (Figure 16). Additionally, the levels of MITF were observed by treatment with PD98059, SP600125, and SB203580, and the protein levels that had decreased by Bay 11-7082 were confirmed to increase again (Figure 17). However, p38 inhibitor treatment did not increase protein levels again. These results showed that the increase in melanin due to p38 activation is independent of MITF expression and occurs through a mechanism different from the one leading to increased MITF expression.

Anti-inflammatory effect by Bay 11-7082 in K14-SCF transgenic mice.

Hair was removed on the dorsal and abdomen of K14-SCF transgenic mice, and after 2 days, the abdominal skin was sensitized by treatment with 100 μl of 0.5% DNFB. Afterward, on days 3, 5, and 7, the dorsal skin was treated with 100 μl each of 0.1% DNFB and Bay 11-7082 (30 μM), and on days 9, 11, and 13, the animals were treated with 30 μM of Bay 11-7082 and then sacrificed on day 15. (Figure 18A). As a result, it was confirmed that inflammation induced by DNFB was restored by Bay 11-7082 (Figure 18B).

To confirm the anti-inflammatory effect of Bay 11-7082, part of the inflamed skin tissue was cut and subjected to Hematoxylin and eosin (H&E) staining. As a result of H&E staining, it was confirmed that the epidermis of mice treated with DNFB was thickened compared to the control group, and the number of inflammatory cells was also increased. In mice treated with Bay 11-7082 on the skin where inflammation was induced by DNFB, the thickness of the epidermis was confirmed to be relatively thinner compared to mice treated only with DNFB, and inflammatory cells were also reduced (Figure 18C). To confirm whether the inflammatory cytokines increased by DNFB were decreased by Bay 11-7082, the mRNA of the inflammatory cytokines was confirmed. As a result, the mRNA of inflammatory cytokines increased by DNFB was decreased by Bay 11-7082 (Figure 19).

Anti-melanogenic effect by Bay 11-7082 in K14-SCF transgenic mice.

After treating the ears and tail of K14-SCF transgenic mice with 100 μl of Bay 11-7082 (30 μM) every other day for 35 days, part of the ear and tail tissue was cut to confirm the effect of Bay 11-7082. As a result, a melanin reduction of approximately 30% was seen in the ear and tail tissue treated with Bay 11-7082 compared to the control group (Figure 20). Then Fontana Masson staining was performed. As a result of Fontana Masson staining, melanin was reduced by about 30% when treated with Bay 11-7082 compared to the control group, and this was expressed as the melanin index (Figure 21).

Anti-melanogenic effect by Bay 11-7082 after induction of inflammation in K14-SCF transgenic mice.

Hair was removed on the dorsal and abdomen of K14-SCF transgenic mice, and after 2 days, the abdominal skin was sensitized by treating it with 100 μl of 0.5% DNFB. Afterward, on days 3, 5, and 7, the dorsal skin was treated with 100 μl each of 0.1% DNFB and Bay 11-7082 (30 μM). After that, the dorsal skin was treated with 30 μM of Bay 11-7082 every other day for 27 days (Figure 22A). To confirm

the anti-inflammatory effect of Bay 11-7082, part of the skin tissue, including the area where inflammation occurred, was cut. As a result, it was confirmed that melanin increased in mice treated with DNFB compared to the control group, and it was confirmed that Bay 11-7082 decreased melanin increased due to DNFB (Figure 22B). Then Fontana Masson staining was performed. As a result of Fontana Masson staining, it was confirmed that melanin increased significantly when treated with DNFB compared to the control group, and a decrease in melanin of about 25% was confirmed when treated with Bay 11-7082. It was expressed as the melanin index (Figure 22C, D).

Anti-melanogenic effect by Bay 11-7082 in co-culture of human keratinocytes and melanocytes and *ex vivo* human skin culture.

The anti-melanogenic effect of Bay 11-7082 was confirmed in Mel-ab cells. To confirm whether this anti-melanogenic effect can be applied to human skin, the toxicity of Bay 11-7082 was confirmed in normal human melanocytes (NHM), and it was observed to be non-toxic up to 5 μM (Figure 23). Bay 11-7082 was treated with non-toxic concentration in NHM cells for 72 h, and melanin content decreased in a concentration-dependent manner. At 5 μM, melanin decreased by about 40% (Figure 24). Since Bay 11-7082 reduced melanin synthesis, it was expected to reduce the activity of tyrosinase, which directly affects melanin synthesis, and NHM cells were treated with Bay 11-7082 at 5uM for 72 h, and then tyrosinase activity was confirmed. As expected, about 30% of tyrosinase activity was inhibited at 5 μM (Figure 25).

To confirm whether Bay 11-7082 can be applied to clinical research to reduce melanin, normal human melanocytes and human keratinocytes were co-cultured and treated with 10ng/mL stem cell factor (SCF) and 0.1nM endothelin-1 (ET-1). And Bay 11-7082 (5 μM) was treated for 72 hours. Afterward, the melanin content was confirmed, and it was confirmed that melanin increased by 20% with SCF and ET-1, and that melanin decreased when treated with Bay 11-7082 (Figure 26).

Additionally, an *ex vivo* human skin organ culture was performed. Bay 11-7082 (30 μM) was applied to the tissue, and the medium was treated with Bay 11-7082 (15 μM) and treated with UVB 220 mJ/ cm^2 for 96 h. Fontana Masson staining confirmed that melanin increased in UVB-treated human skin compared to the DMSO-treated control group, and melanin was confirmed to be reduced by treatment with Bay 11-7082. This was expressed as the melanin index (Figure 27).

Discussion

Although numerous studies have been developed to treat post-inflammatory hyperpigmentation (PIH), there is still no perfect treatment method. Moreover, there is a lack of understanding of PIH's genesis mechanism and underlying physiological causal relationship. A molecular-level understanding of how inflammation affects melanin synthesis and how melanin synthesis changes after inflammation is still unknown and requires more research.

In general, reducing inflammation also suppresses melanin synthesis. This is because there is often an interaction between inflammation and melanin synthesis. However, some treatments reduce inflammation but increase melanin, causing side effects. For example, glucocorticoid is a therapeutic agent that exhibits anti-inflammatory effects in the 1940s and is still widely used to treat acute and chronic inflammation, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and eczema [29]. However, this treatment is known to increase melanin in B16 melanoma cells [30]. In addition, salicylamide, a non-steroidal anti-inflammatory agent, is used as an analgesic and antipyretic but is known to increase melanin synthesis in B16F1 cells [31]. Therefore, these agents may have side effects due to melanin synthesis. Therefore, we analyzed the pathogenesis of post-inflammatory pigmentation, understood the interaction between inflammation and melanin synthesis, and studied an effective drug that simultaneously reduces inflammation and melanin.

In this study, we screened drugs showing anti-melanogenic effects using the NF-κB-Luciferase Reporter (Luc) - Jurkat Cell Line to find drugs that regulate the NF-κB signaling pathway, an important transcription factor widely involved in the regulation of apoptosis, inflammation, immunity, and cell proliferation. As a result, Bay 11-7082 was selected as it was confirmed to reduce NF-κB-luciferase (NF-κB-Luc) reporter genes the most. The selected Bay 11-7082 is known to be an NF-κB inhibitor and was confirmed to reduce various inflammatory cytokines by inhibiting NF-κB through Raw 264.7 and Hacat cells. Additionally, a decrease in melanin content and melanin biosynthesis-related proteins was confirmed in Mel-ab cells and normal human melanocytes by Bay 11-7082. As a result, it was confirmed that Bay 11-7082 inhibits inflammation and melanin synthesis.

As in vitro, a decrease in melanin and inflammation was confirmed in vivo. When Bay 11-7082 was treated alone on the ears and tail of the K14-SCF mice, a decrease in melanin was confirmed, and on the dorsal, inflammation was induced with DNFB, and inflammation was confirmed to be reduced by

Bay 11-7082. Additionally, it was confirmed that inflammation and melanin decreased simultaneously by Bay 11-7082 in mice.

In addition, a co-culture of normal human melanocytes and keratinocytes was performed by treating them with SCF/ET-1, a substance stimulated by ultraviolet rays. It was confirmed that the increased melanin synthesis caused by the treated irritant was suppressed, and in ex vivo human skin tissue, melanin increased by ultraviolet rays was reduced. It shows inflammation and melanin reduction in vitro, in vivo, and ex vivo, which means that Bay 11-7082 can be used as an agent for PIH by suppressing the activation of inflammation that causes PIH.

However, although this study demonstrated that Bay 11-7082 suppresses inflammation and melanin synthesis, the exact mechanism by which inflammation and melanin are simultaneously suppressed has not been revealed. Additionally, it is necessary to analyze changes in melanin-related synthetic proteins following melanin reduction due to Bay 11-7082 treatment in mice skin and *ex vivo* human skin. Bay 11-7082 is highly toxic, so high efficacy can only be expected if it passes through the skin layer in low concentrations. Therefore, follow-up studies such as permeability will be necessary to confirm whether Bay 11-7082 can be effective on the skin.

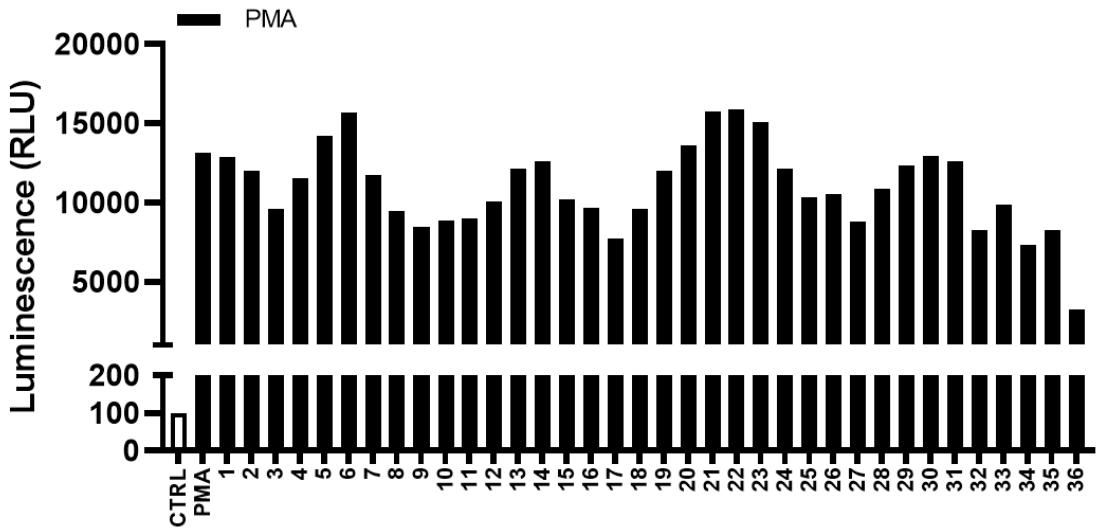


Figure 1. Comparison of NF-κB- luciferase (NF-κB-Luc) reporter genes inhibition by drug with anti-melanogenic effect in NF-κB-Luciferase Reporter (Luc) - Jurkat Cell.

NF-kB Luciferase reporter Jurkat stable cells were treated with screening drug (1 μM) and PMA (10 nM) for 4 hours. After 4 h, the Assay mixture and Luciferin were dispensed into the culture medium. Luminescence was measured using a microplate reader.

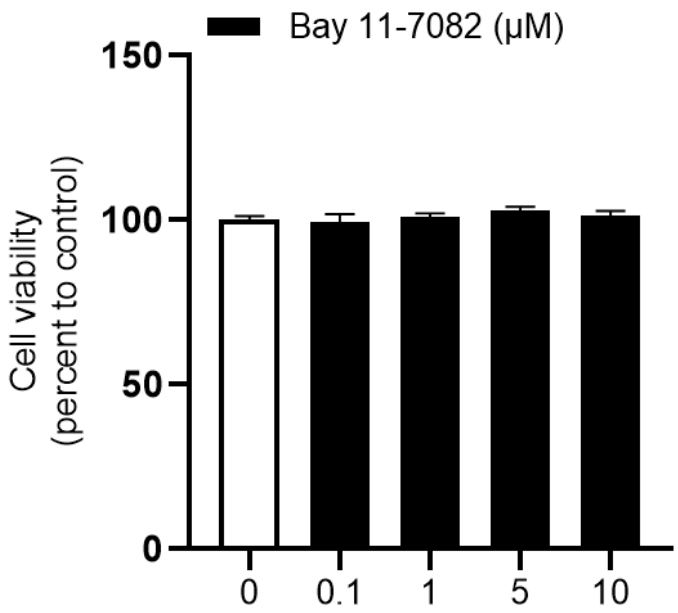


Figure 2. Cytotoxicity evaluation according to treatment with different concentrations of Bay 11-7082 in Raw 264.7 cells.

Raw 264.7 cells were treated with Bay 11-7082 at 0.1 - 10 μM for 24 h, and then cytotoxicity was assessed through an MTT cell proliferation assay. The control group was treated with DMSO, and a graph was drawn using this value as the standard.

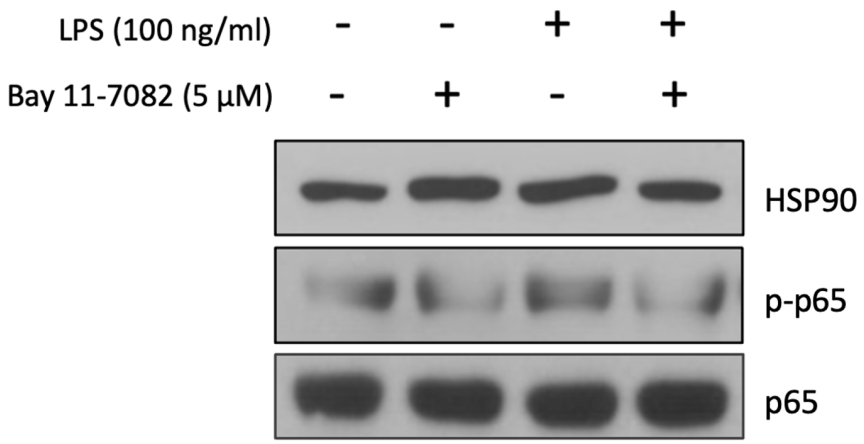


Figure 3. Changes of p65 protein expression in Raw 264.7 cells following Bay 11-7082 treatment.

Raw 264.7 cells were pre-treated with Bay 11-7082 (5 μM) for 1 hour. Subsequently, Raw 264.7 cells were exposed to LPS (lipopolysaccharide) at a concentration of 1 ug/ml and incubated for 2 h. After the specified incubation periods, the cells were harvested and lysed using a 1% SDS lysis buffer. Western blot analysis was then conducted. The expression of proteins involved in melanin synthesis was assessed using specific antibodies. HSP90α/β was used as an internal loading control.

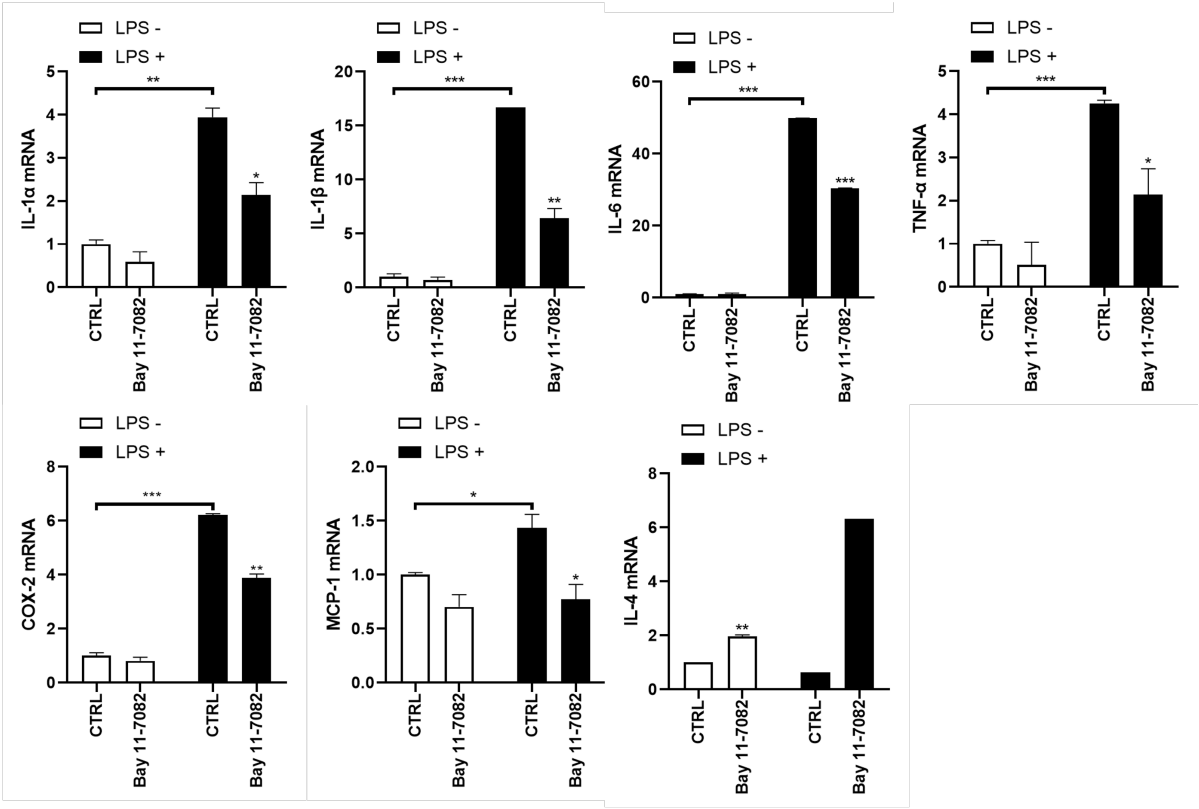


Figure 4. Comparison of changes in mRNA expression levels of inflammatory cytokines in Raw 264.7 cells following Bay 11-7082 treatment.

Raw 264.7 cells were pre-treated with Bay 11-7082 (5 μM) for 1 h. Subsequently, Raw 264.7 cells were exposed to LPS (lipopolysaccharide) at a concentration of 100 ng/ml and incubated for 2 h. After the specified incubation period, RNA was extracted, cDNA was synthesized, and mRNA expression of inflammatory cytokines was analyzed using RT-qPCR.

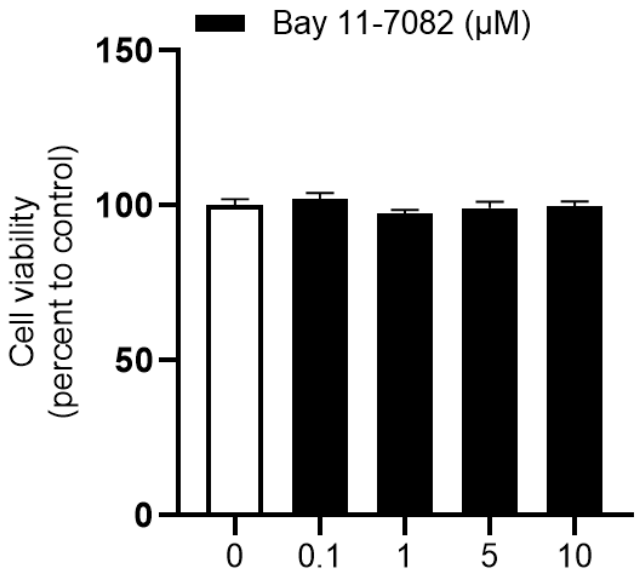


Figure 5. Cytotoxicity evaluation according to treatment with different concentrations of Bay 11-7082 in Hacat cells.

Hacat cells were treated with Bay 11-7082 at 0.1 - 10 μM for 24 h, and then cytotoxicity was assessed through an MTT cell proliferation assay. The control group was treated with DMSO, and a graph was drawn using this value as the standard.

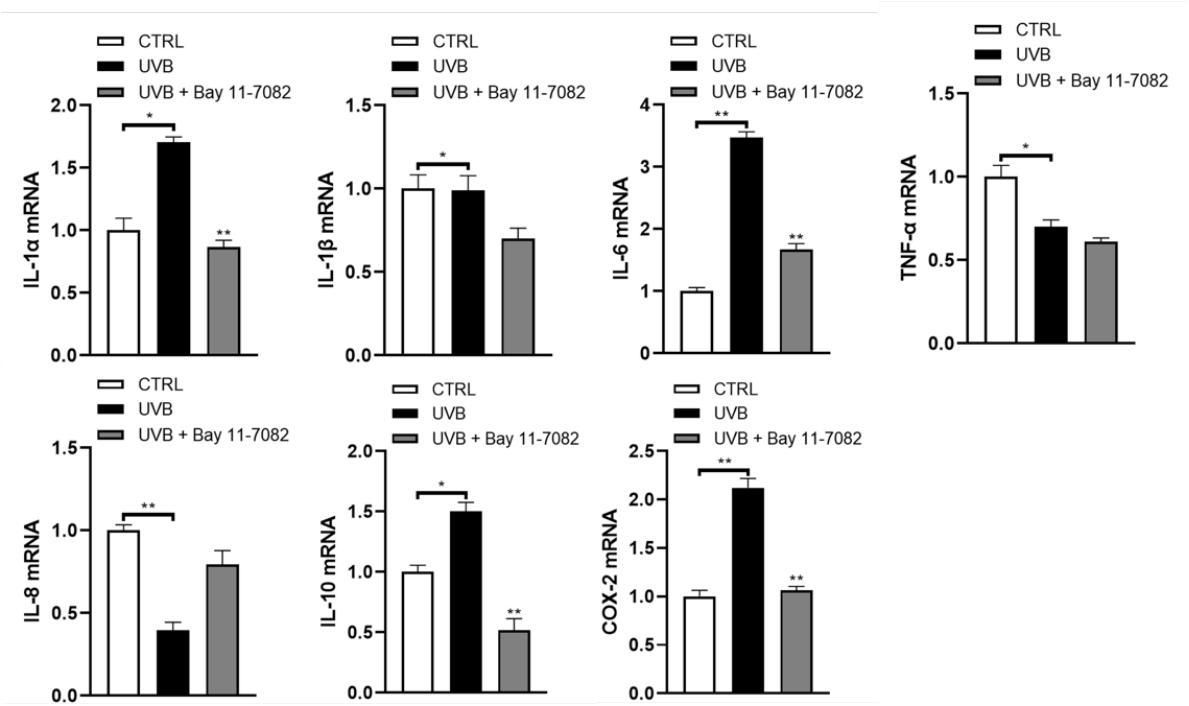


Figure 6. Comparison of changes in mRNA expression levels of inflammatory cytokines in Hacat cells following Bay 11-7082 treatment.

Hacat cells were pre-treated with Bay 11-7082 (5 μM) for 1 h. Subsequently, Hacat cells were exposed to UVB (100 mJ/ cm^2). After 2 h, RNA was extracted, cDNA was synthesized, and mRNA expression of inflammatory cytokines was analyzed using RT-qPCR.

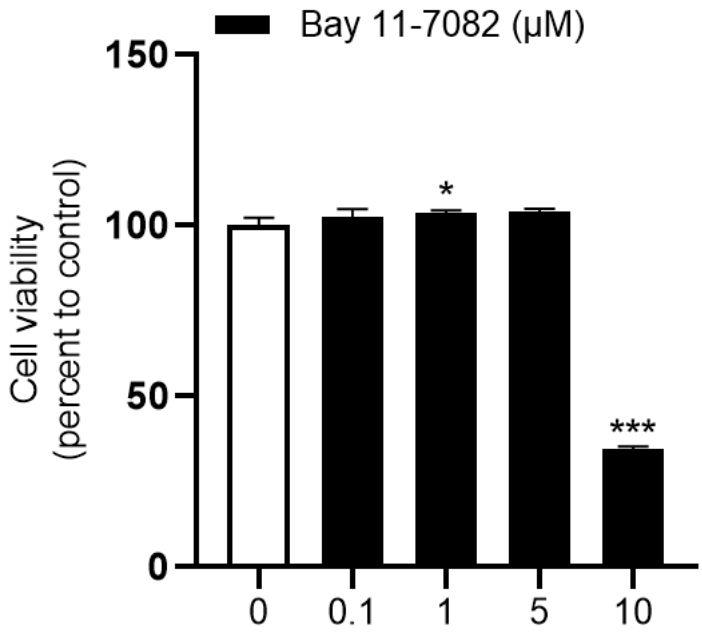


Figure 7. Cytotoxicity evaluation of Mel-ab cells following treatment with different concentrations of Bay 11-7082.

Mel-ab cells were treated with 0.1 – 10 μM concentrations of Bay 11-7082 for 72 h. Cytotoxicity was assessed using the MTT cell proliferation assay. The control group was treated with DMSO, and this was used as the standard for comparison when creating the graphs.

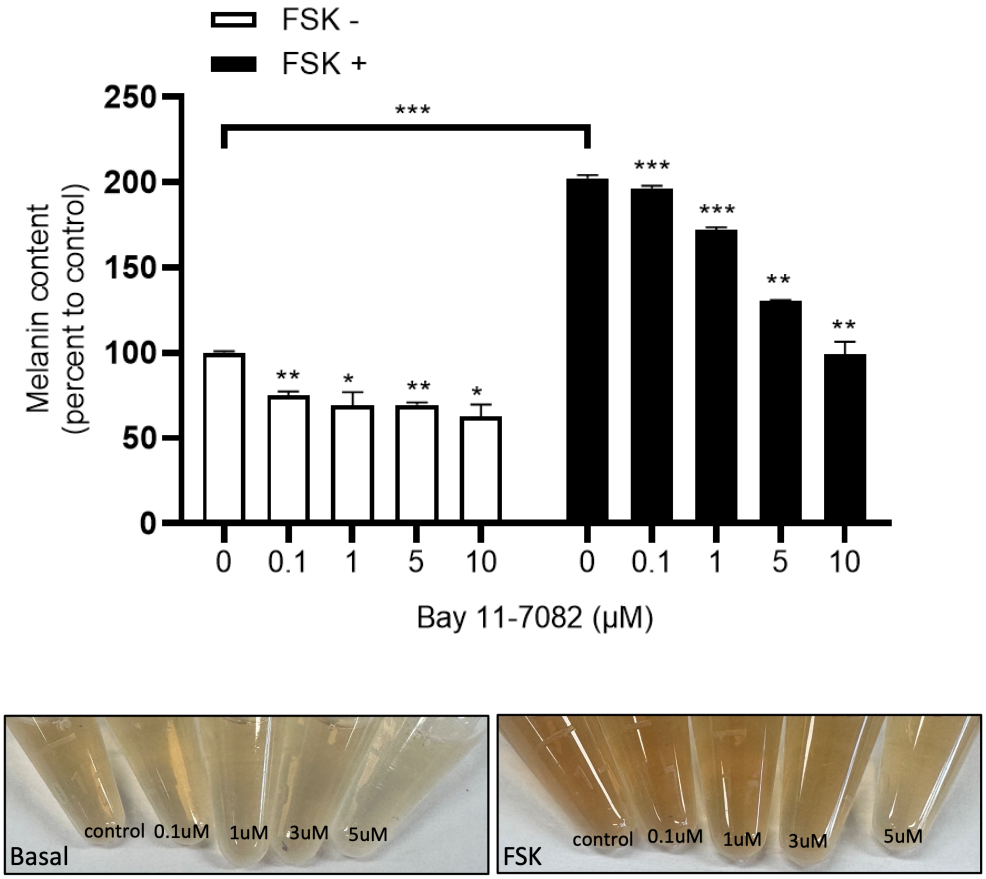


Figure 8. Measurement of melanin content in Mel-ab cells following treatment with different concentrations of FSK and Bay 11-7082.

Mel-ab cells were pre-treated with different concentrations of Bay 11-7082 for 1 h and then exposed to FSK (10 μM) for 72 h. The cells were lysed with 1N NaOH, and absorbance was measured at 405nm. Quantification was performed using the BCA assay, and melanin content per unit protein was graphed.

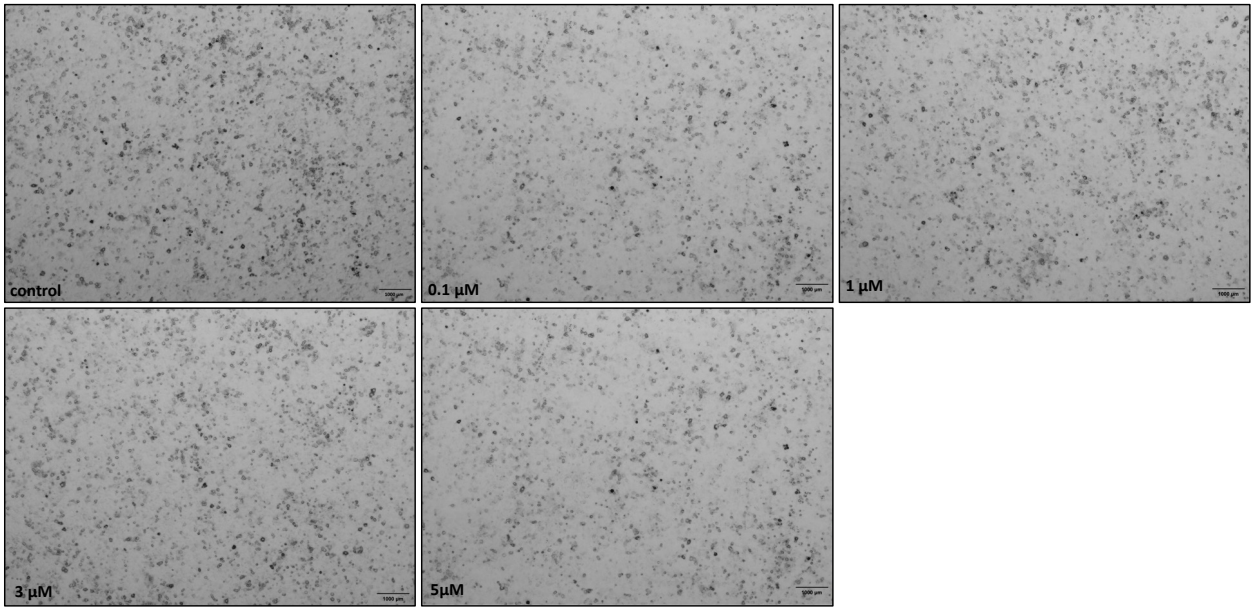


Figure 9. Microscopic observation of melanin content in Mel-ab cells following treatment with different concentrations of Bay 11-7082.

Mel-ab cells were treated with different concentrations of Bay 11-7082 for 72 h, and melanin content was observed using the microscope.

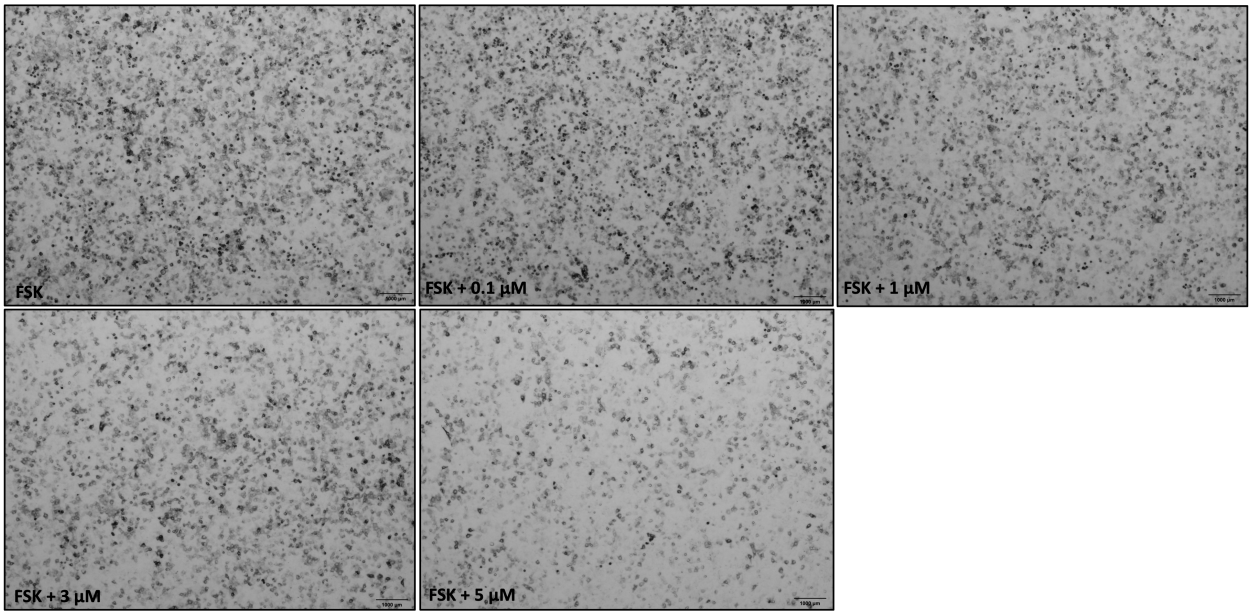


Figure 10. Microscopic observation of melanin content in Mel-ab cells following treatment with different concentrations of FSK and Bay 11-7082.

Mel-ab cells were pre-treated with Bay 11-7082 at different concentrations for 1 h and treated by FSK (10 μM) for 72 h. Melanin content was observed in the cells using the microscope.

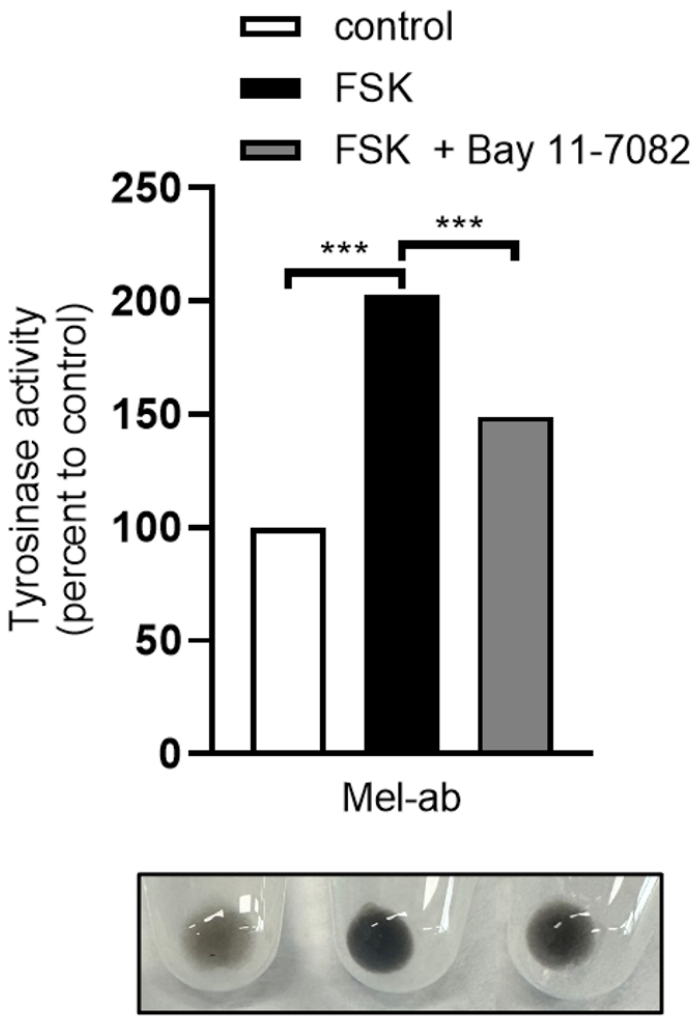


Figure 11. Assessment of tyrosinase activity by treatment of Bay 11-7082 and FSK in Mel-ab cells.

Mel-ab cells were treated with Bay 11-7082 (5 μM) and FSK (10 μM) for 72 hours. Subsequently, the cells were lysed using Tyrosinase lysis buffer and subjected to freeze/thaw cycles. After quantifying the protein concentration using the BCA assay, the cell lysates were mixed with L-DOPA and incubated at 37°C for 1 h. Tyrosinase activity was measured at an absorbance of 475 nm.

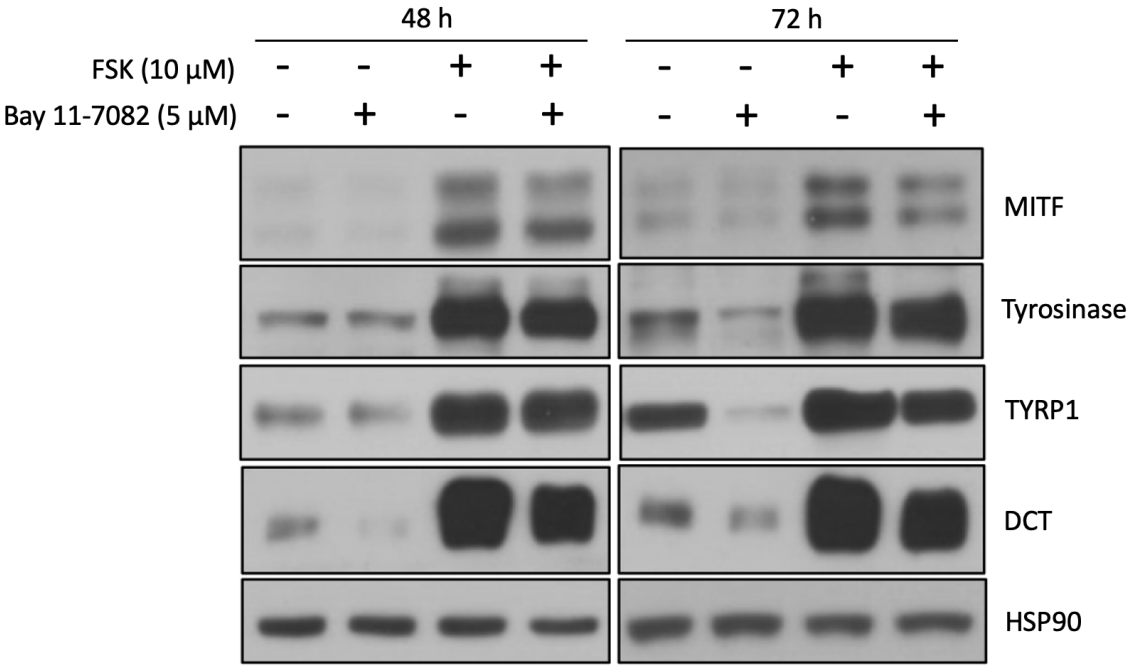


Figure 12. Comparison of melanogenesis-involved protein expression by treatment of Bay 11-7082 and FSK in Mel-ab cells

Mel-ab cells were treated with Bay 11-7082 (5 μM) for 1 h and then treated with FSK (10 μM). After treatment for 48h and 72 h, cells were lysed with 1% SDS lysis buffer, and a western blot was performed. The expression of proteins involved in melanin synthesis was confirmed using each antibody, and HSP90α/β was used as an internal loading control.

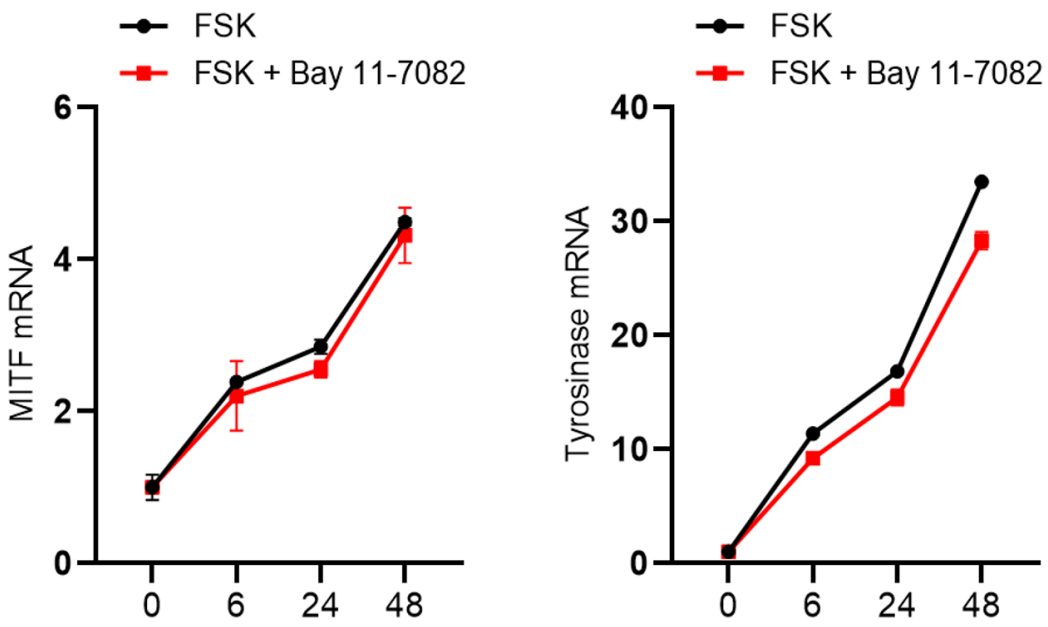


Figure 13. Comparison of changes in mRNA expression levels of melanogenic genes by treatment of Bay 11-7082 and FSK in Mel-ab cells.

Mel-ab cells were pre-treated with Bay 11-7082 (5 μM) for 1 h before treatment with FSK (10 μM). Cells were harvested at the indicated times. Afterward, RNA was extracted, cDNA was synthesized, and RNA of genes related to melanin synthesis was analyzed using RT-qPCR.

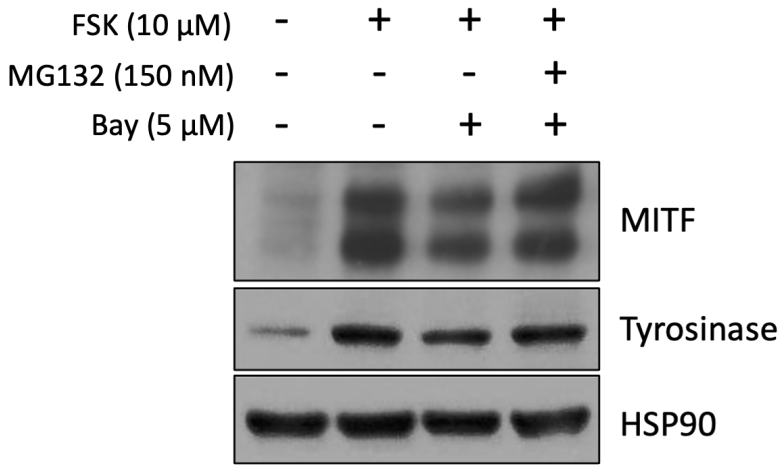


Figure 14. Comparison of melanogenesis-involved protein expression by Bay 11-7082 and MG132 treatment in Mel-ab cells.

Mel-ab cells were pre-treated with FSK (10 μM) for 12 h, and then MG132 (80 nM) was pre-treated for 2 h, and Bay 11-7082 (5 μM) was treated. After 48 h, cells were lysed with 1% SDS lysis buffer, and a western blot was performed. The expression of proteins involved in melanin biosynthesis was confirmed using each antibody, and HSP90α/β was used as an internal loading contr

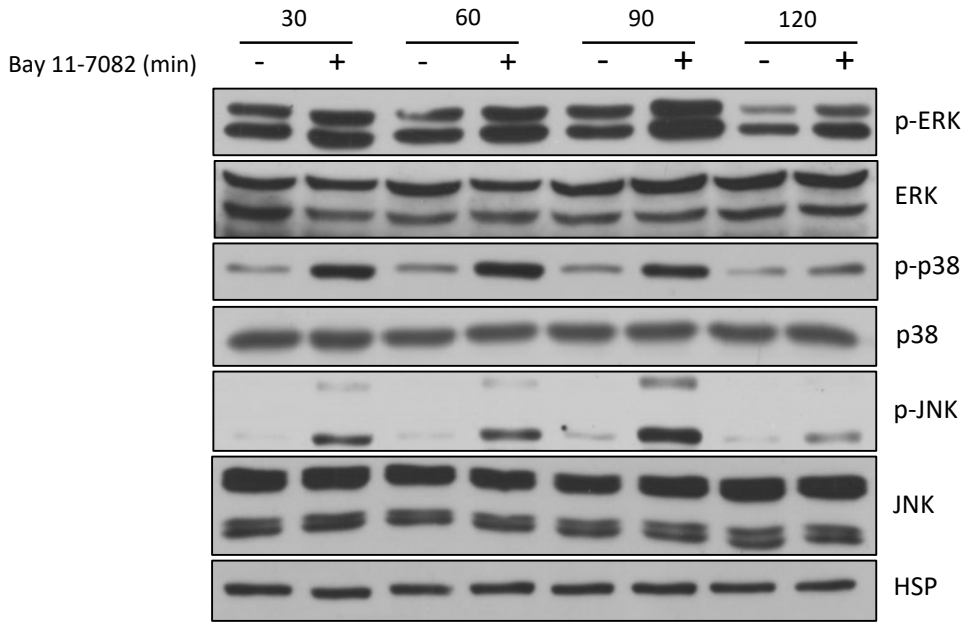


Figure 15. Phosphorylation changes following Bay 11-7082 treatment in Mel-ab cells.

Mel-ab cells were pre-treated with Bay 11-7082 (5 μM) for 1 hour and then treated with FSK (10 μM). Cells were lysed with 1% SDS lysis buffer at different times and a western blot was performed. HSP90α/β was used as an internal loading control.

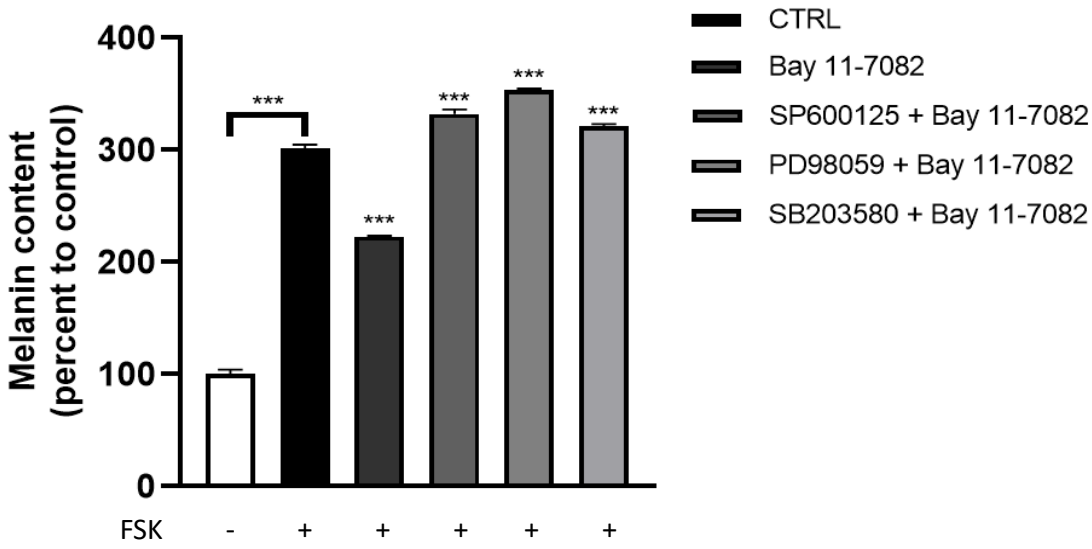


Figure 16. Changes in melanin content when inhibiting of activation JNK, ERK, and P38.

Mel-ab cells were pre-treated with SP600125 (JNK inhibitor, 15 μM), PD98059 (ERK inhibitor, 5 μM) and SB203580 (P38 inhibitor, 10 μM) for 1 h and then treated with Bay 11-7082 (5 μM) and FSK (10 μM). After 72 h, cells were lysed with 1N NaOH and absorbance was measured at 405 nm. This was quantified using a BCA assay, and the melanin content per unit protein was graphed.

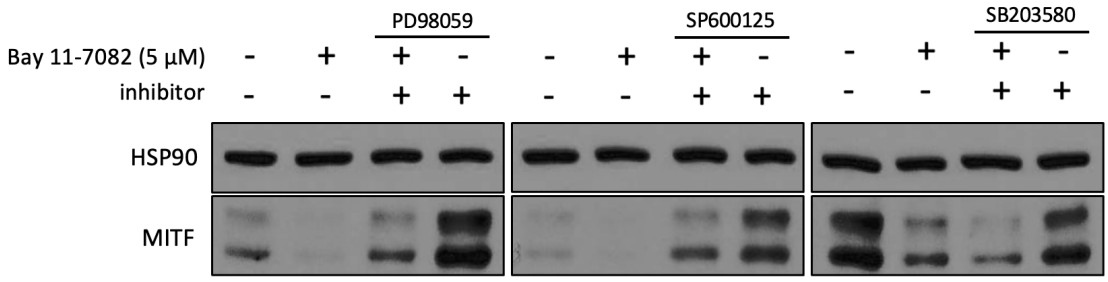


Figure 17. Changes in the levels of melanogenesis-involved proteins when inhibiting ERK, JNK, and P38 activation, a melanin reduction pathway of Bay 11-7082.

Mel-ab cells were pre-treated with PD98059 (10 μM), SP600125 (15 μM) and SB203580 (10 μM) for 1 hour and then treated with Bay 11-7082 (5 μM). After 6 h, cells were lysed with 1% SDS lysis buffer, and a western blot was performed. HSP90α/β was used as an internal loading control.

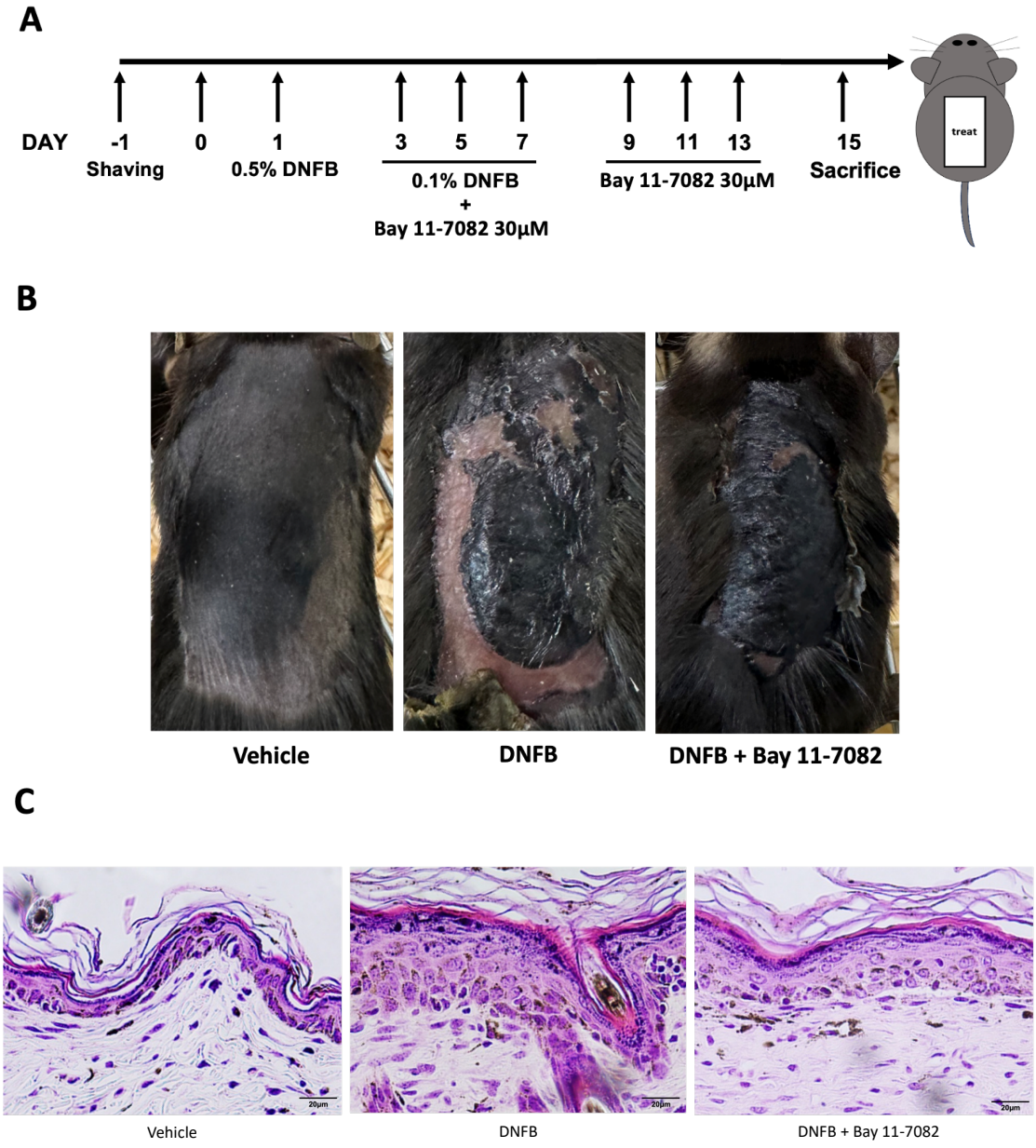


Figure 18. Changes in skin inflammation according to treatment with DNFB and Bay 11-7082 in K14-SCF mice.

After sensitizing the abdomen of the K14-SCF mice with 0.5% DNFB, it was treated with 0.1% DNFB and Bay 11-7082 (30 μM) on days 3, 5, and 7. Bay 11-7082 (30 μM) was treated alone on days 9, 11, and 13, and sacrifice was performed on day 15. In the DNFB model, only DNFB was treated in the same manner until day 7 (A). The mice photo was taken on the day 14, and the epidermis and dermis were observed through H&E staining (B, C).

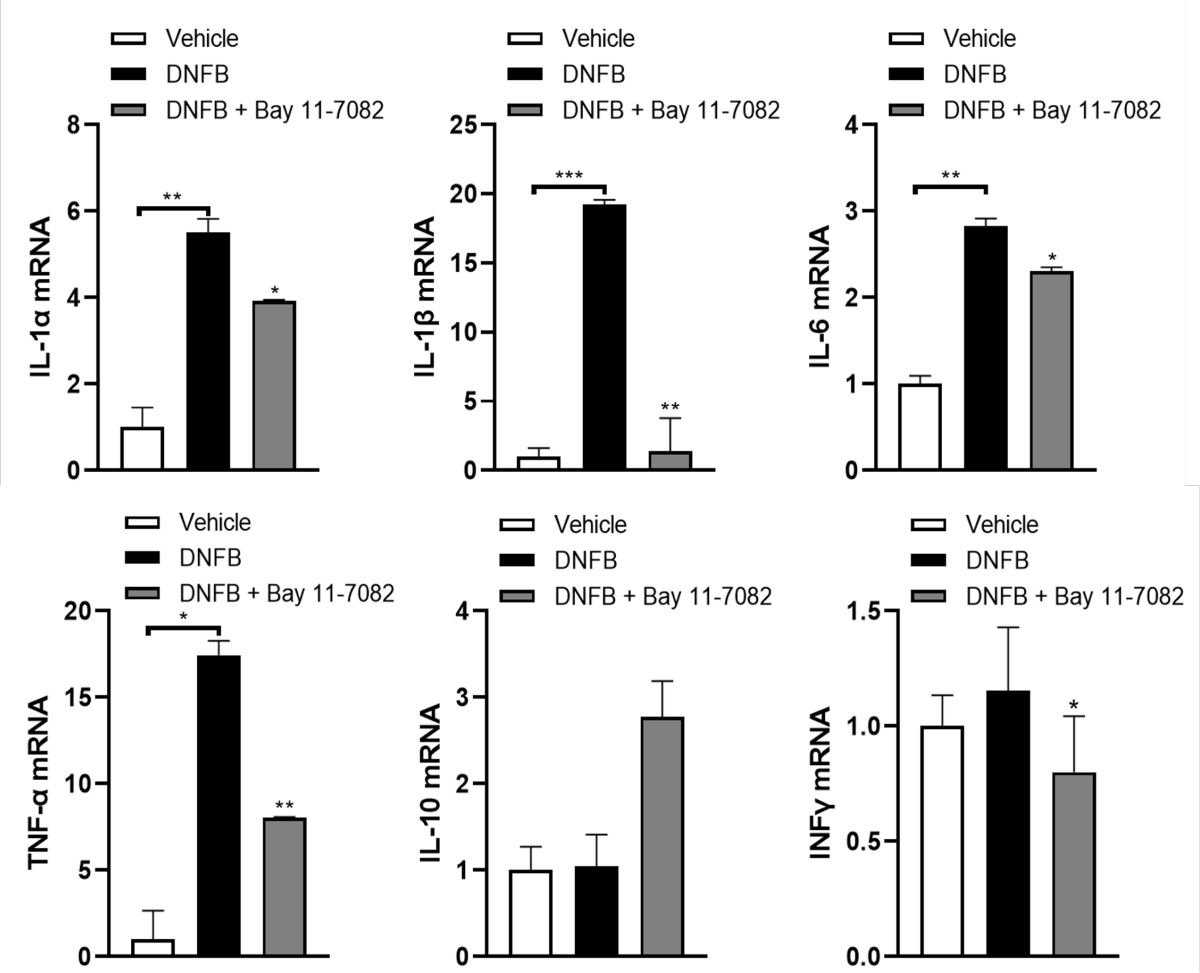


Figure 19. Changes in mRNA of inflammatory cytokines following treatment with DNFB and Bay 11-7082 in K14-SCF mice.

After sensitizing the abdomen of the K14-SCF mouse with 0.5% DNFB, it was treated with 0.1% DNFB and Bay 11-7082 (30 μM) on days 3, 5, and 7, and Bay 11-7082 (30 μM) on days 9, 11, and 13. It was treated alone. A sacrifice was performed on the day 15. In the DNFB model, only DNFB was processed in the same manner until day 7 Afterward, RNA was extracted, cDNA was synthesized, and RNA of genes related to melanogenesis was analyzed using RT-qPCR.

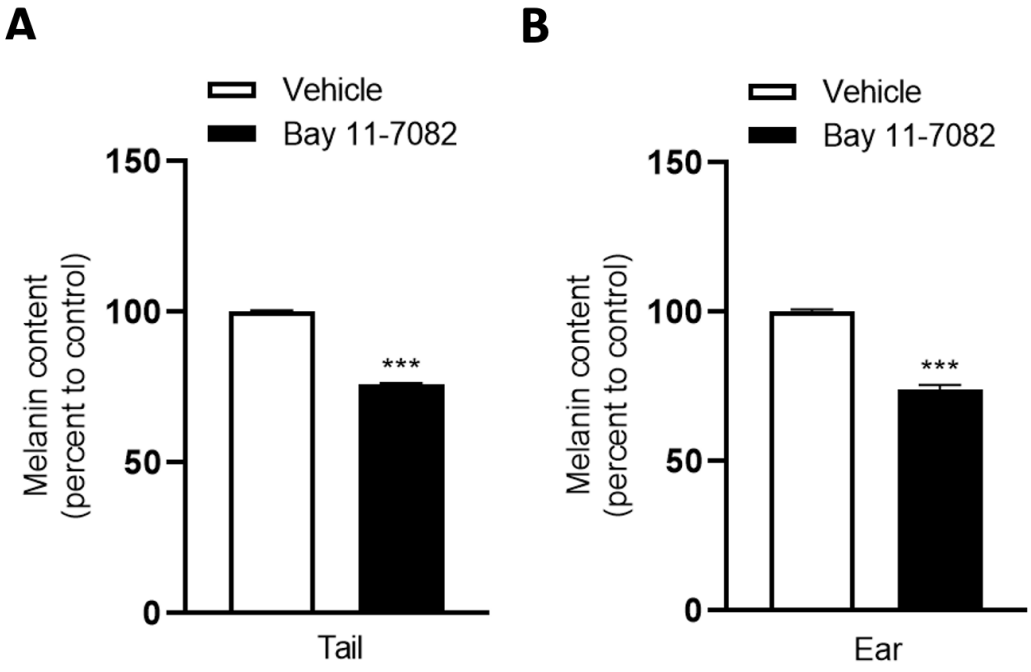


Figure 20. Changes in melanogenesis following treatment with Bay 11-7082 in the ears and tail of K14-SCF mice.

K14-SCF mice was treated with 30 μM of Bay 11-7082 on the tail (A) and ears (B) every other day for 35 days. Afterwards, the tissue was dissolved in 1N NaOH and the absorbance was measured at 405 nm. This was quantified using a BCA assay, and the melanin content per unit protein was graphed.

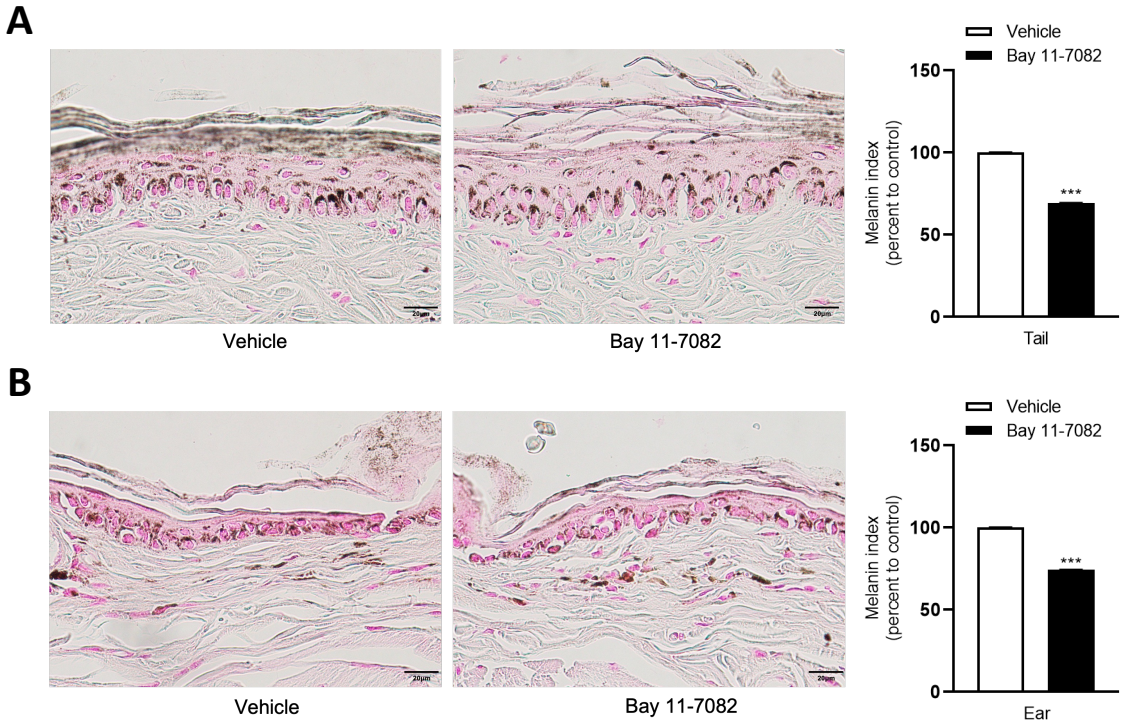


Figure 21. Comparison of melanin content according to treatment with Bay 11-7082 in the ears and tail of K14-SCF mice.

The tail (A) and ears (B) of the K14-SCF mice were treated with 100 μl of Bay 11-7082 (30 μM) every other day for 35 days. The melanin content was expressed as an index using Fontana Masson staining.

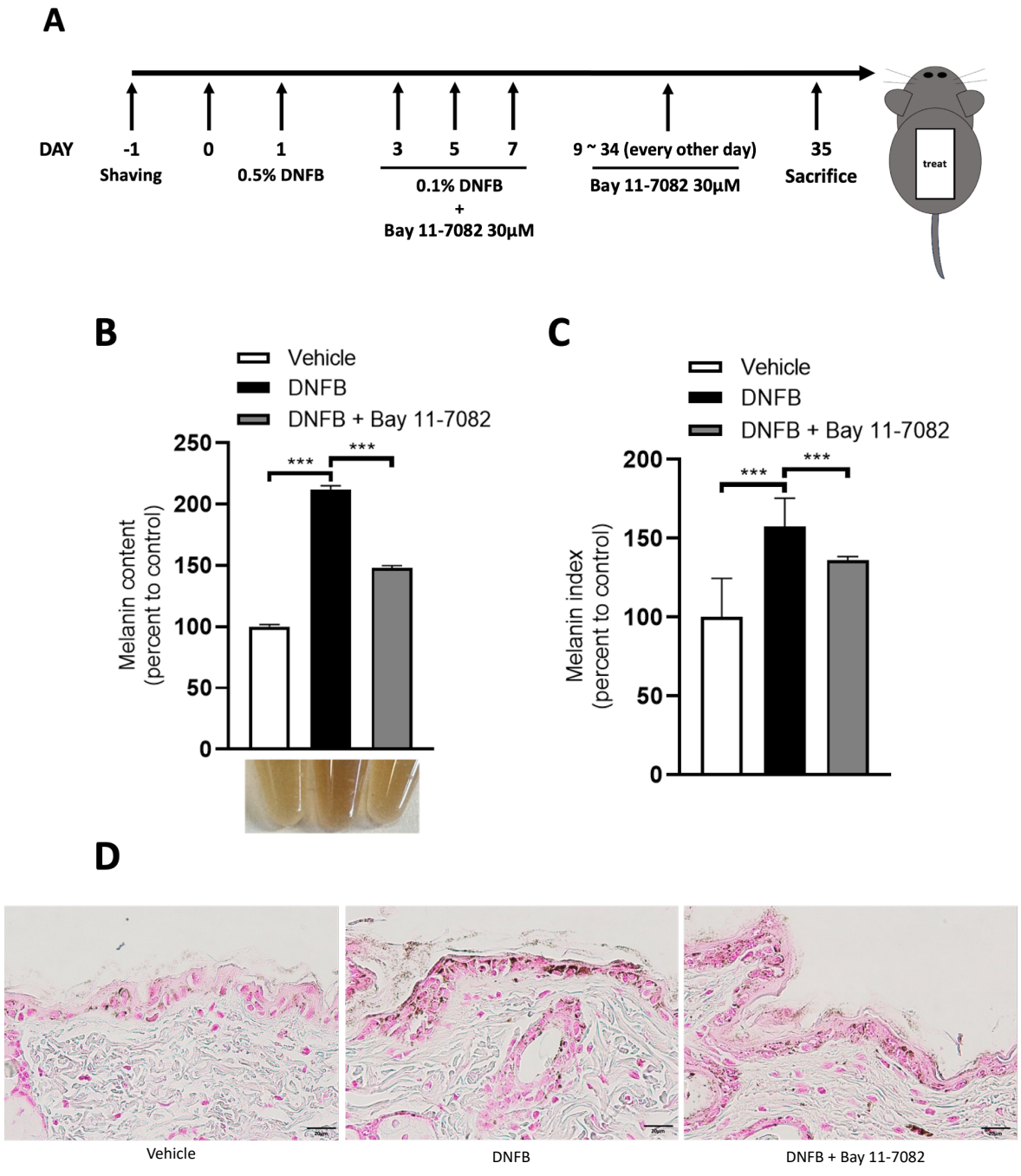


Figure 22. Comparison of melanin content according to treatment with DNFB and Bay 11-7082 in K14-SCF mice.

DNFB and Bay 11-7082 treatment conditions were shown in K14-SCF mice (A). The tissue was dissolved in 1N NaOH and the absorbance was measured at 405 nm. This was quantified using a BCA assay, and the melanin content per unit protein was graphed (B). The melanin content was expressed as an index using Fontana Masson staining (C, D).

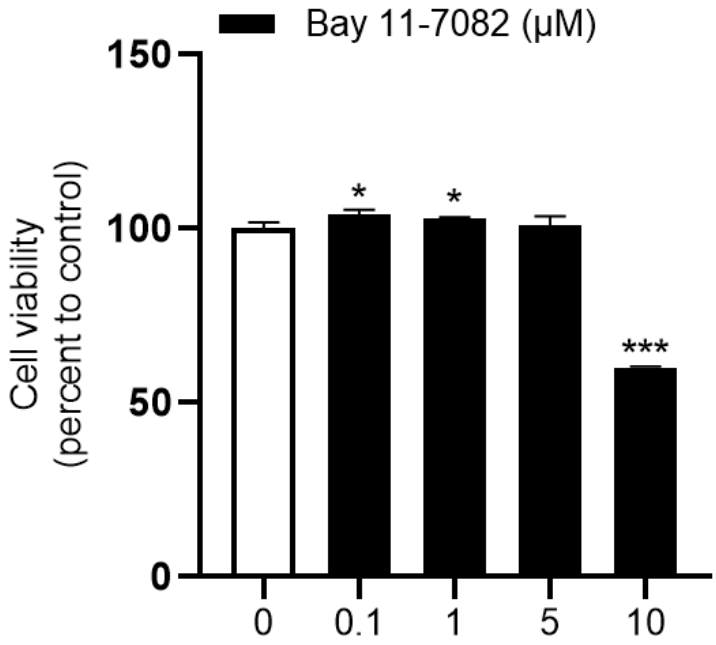


Figure 23. Cytotoxicity evaluation according to treatment with Bay 11-7082 concentrations in normal human melanocytes (NHM).

Normal human melanocytes were treated with Bay 11-7082 at different concentrations for 72 h, and then cytotoxicity was assessed through MTT cell proliferation assay. The control group was treated with DMSO, and a graph was drawn using this value as the standard.

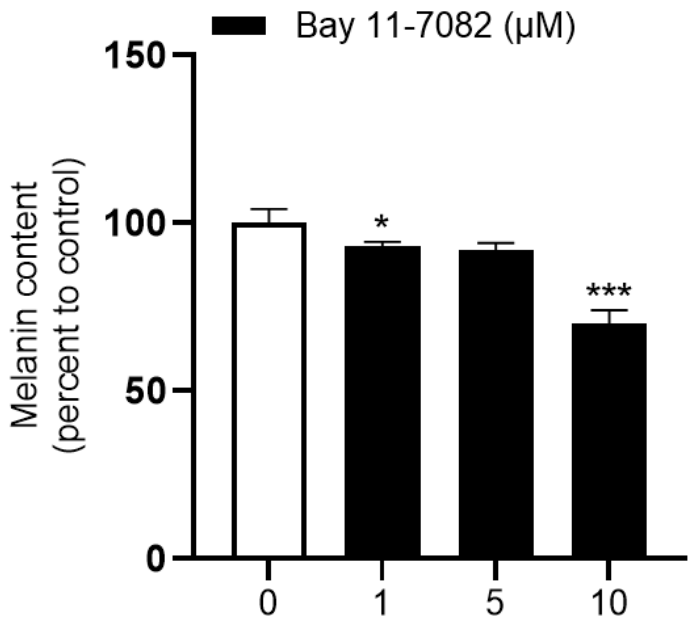


Figure 24. Measurement of melanin content in normal human melanocytes (NHM) according to treatment with different concentrations of Bay 11-7082.

Normal human melanocytes were treated with Bay 11-7082 at different concentrations for 72 h, and then cytotoxicity was assessed through MTT cell proliferation assay. The control group was treated with DMSO, and a graph was drawn using this value as the standard.

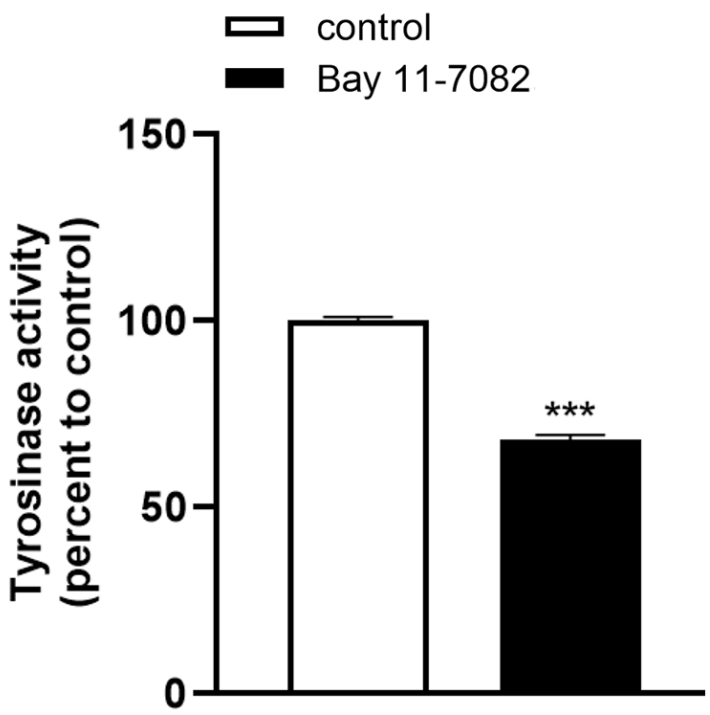


Figure 25. Measurement of tyrosinase activity in normal human melanocytes (NHM) according to treatment with Bay 11-7082.

Normal human melanocytes were treated with Bay 11-7082 (5 μM) for 72 h, and then the cells were lysed with Tyrosinase lysis buffer and subjected to freeze/thaw cycles. After quantification was performed using the BCA assay to adjust the protein concentration, L-DOPA was added to the cell lysate and incubated at 37°C for 1. Tyrosinase activity was measured at absorbance at 475 nm.

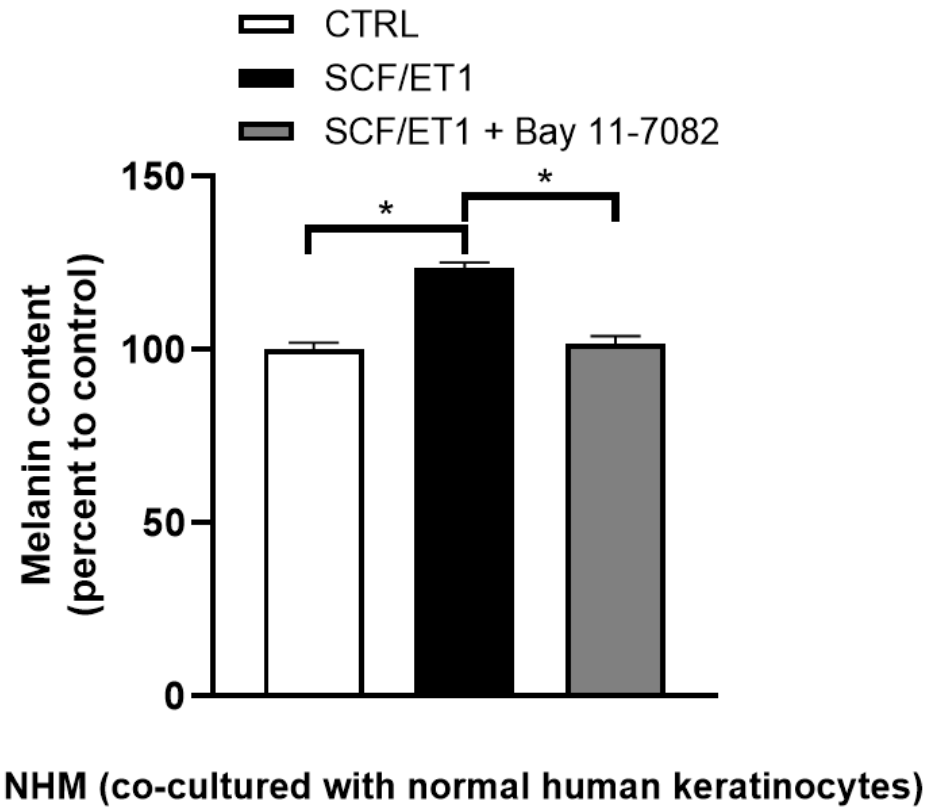
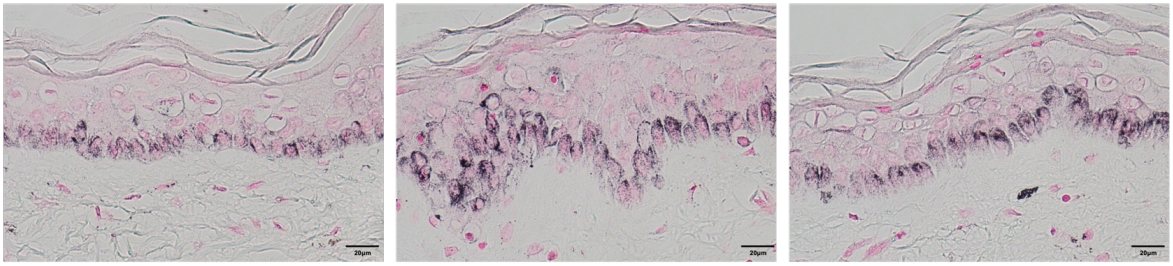


Figure 26. Measurement of melanin content according to Bay 11-7082 treatment in coculture of normal human melanocytes and normal human keratinocytes.

Normal human keratinocytes and normal human melanocytes were cocultured and then treated with 5 μM Bay 11-7082, 10 ng/mL stem cell factor (SCF), and 0.1 nM endothelin-1 (ET-1). After 72 h, cells were lysed with 1N NaOH and absorbance was measured at 405 nm. This was quantified using a BCA assay, and the melanin content per unit protein was graphed.



Vehicle UVB UVB + Bay 11-7082

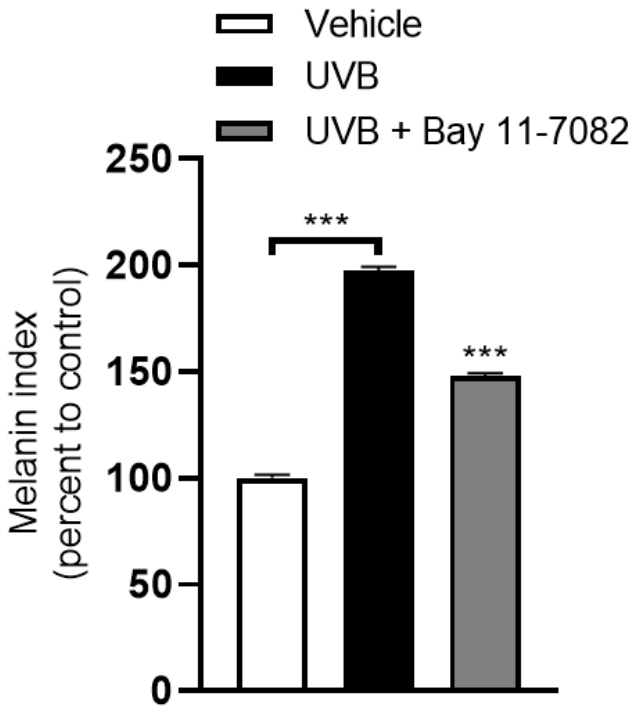


Figure 27. Comparison of melanin content according to UVB and Bay 11-7082 treatment on ex vivo human skin.

ex vivo human skin organ was treated with Bay 11-7082 (30 μM), and Bay 11-7082 (15 μM) was treated with the medium for 96 h and treated with UVB 220 mJ/ cm^2. Melanin content was confirmed using Fontana Masson staining and expressed as an index.

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국문 요약

Post-inflammatory hyperpigmentation (PIH)는 외부로부터 부상 또는 피부질환과 같은 원인으로 피부에 염증이 일어나 색소침착을 일으키는 후천성 색소장애를 말하며 이로 인해 피부 건강과 외모에 영향을 미치는 심각한 문제이다. 이러한 문제를 다루기 위해 다양한 치료법이 개발되어 왔으나, 아직까지도 완전한 치료 방법은 없다. 또한 PIH 의 발생 메커니즘 및 기저 생리학적 인과관계에 대한 이해도 부족하다. 염증이 어떻게 멜라닌 생성에 영향을 미치는지, 그리고 염증 후에 어떻게 멜라닌 생성이 변화하는지에 대한 분자적 이해는 여전히 미지의 영역이다. 따라서 PIH 의 발생 메커니즘을 분석하여 염증과 멜라닌 생성 간의 상호 작용을 이해하고 염증 억제와 멜라닌 생성 억제를 동시에 조절하는 효과적인 치료법의 개발을 목표로 한다.

UVB 는 햇빛에서 나오는 자외선 중 하나이며 멜라닌 생성에 관여하는 MITF 와 그 하위 유전자인 Tyrosinase, Tyrp-1, DCT 등을 촉진시킨다. 본 연구는 Bay 11-7082 가 melanocytes 의 멜라닌을 억제한다는 것을 확인하였으며 Bay 11-7082 가 Forskolin (FSK) 의해 증가된 cellular tyrosinase activity 를 억제하였고 MAPK 인산화를 증가시켜 MITF, Tyrosinase, DCT, Tyrp-1 의 수준을 감소시키는 것을 확인하였다. 또한 immortalized human keratinocytes 인 Hacat 세포와 대식세포인 Raw264.7 세포의 염증성 사이토카인 IL-1a, IL-1b, IL-6, TNF-a, COX-2, MCP-1 등 mRNA 수준을 억제하였고 mice 에 DNFB 로 염증을 유도해 색소침착을 일으켜 Bay 11-7082 의해 염증과 멜라닌을 감소시킨 것을 확인하였다. 또한, Bay 11-7082 에 의해 *ex vivo* Human skin 에 UVB 로 증가된 멜라닌을 감소시켰다.

결과적으로 Bay 11-7082 는 NF-κB 억제제로 염증성 사이토카인을 억제해 염증을 감소시켰고 MAPK/MITF pathway 을 통해 멜라닌을 억제하는 것을 보아 PIH 치료제 개발에 있어 염증과 멜라닌을 동시에 억제할 수 있는 약물의 가능성이 있는 것으로 판단된다.

(중심단어) Post-inflammatory hyperpigmentation (PIH), Bay 11-7082, MAPK/MITF pathway, Melanogenesis